

# **Ecosystem carbon change of shrub-encroached grasslands across a precipitation gradient in South Africa**

**by**

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1 **PREFACE**

2 The research contained in this thesis was completed by the candidate while based in the  
3 Discipline of Ecology, School of Life Sciences of the College of Agriculture, Engineering and  
4 Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research was  
5 financially supported by NRF.  
6

7 The contents of this work have not been submitted in any form to another university and,  
8 except where the work of others is acknowledged in the text, the results reported are due to  
9 investigations by the candidate.  
10  
11

12 

13 Signed: Professor David Ward

14 Date: 03 January 2016  
15

## DECLARATION 1: PLAGIARISM

I, Admore Mureva, declare that:

- (i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;
- (ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;
- (iii) this dissertation does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;
- (iv) this dissertation does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
  - a) their words have been re-written but the general information attributed to them has been referenced;
  - b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced;
- (v) where I have used material for which publications followed, I have indicated in detail my role in the work;
- (vi) this dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;
- (vii) this dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.



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Date: 04 January 2016

## DECLARATION 2: PUBLICATIONS

My role in each paper and presentation is indicated. The \* indicates corresponding author.

### Chapter 2

Mureva, A.\* and Ward D. **Spatial patterns of encroaching shrub species under different grazing regimes in a semi-arid savanna (Eastern Karoo, South Africa)**. Provisionally accepted in the *African Journal of Range and Forage Science*.

A.M. conceived the study presented, collected and analysed the data and wrote the paper. D.W contributed substantially to experimental design, data analysis and manuscript preparation.

### Chapter 3

Mureva, A.\* , Pillay, T., Chivenge, P., Ward, D. and Cramer, M. **Woody plant encroachment enhances carbon sequestration in semi-arid regions and decreases sequestration in humid regions of South Africa**. Formatted for *Nature*.

A.M. conceived the study presented, collected and analysed the data and wrote the paper. TP, PC, DW and MC contributed substantially to experimental design, data analysis and manuscript preparation.

### Chapter 4


Mureva, A.\* , Chivenge, P. and Ward, D., **Storage of carbon and nitrogen in soil physical fractions following woody plant invasion of grassland**. Formatted for *Plant and Soil*.

A.M. conceived the study presented, collected and analysed the data and wrote the paper. PC and DW contributed substantially to experimental design, data analysis and manuscript preparation.

**Chapter 5**

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encroached grasslands along a precipitation gradient.** Formatted for *Soil Biology and  
Biogeochemistry*.

A.M. conceived the study presented, collected, analysed the data and wrote the manuscript. D.W  
contributed substantially to experimental design, data analysis and manuscript preparation.



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Date: 04 January 2016

## ABSTRACT

Grasslands and savannas are experiencing intensive land-cover change due to woody plant encroachment. In South Africa, encroachment affects more than 10 million ha, which is about 8% of the land area. Although a number of factors such as heavy grazing, fire intensity and frequency, soil moisture, nutrients and global climate change have been attributed to grassland invasion by woody plants, the mechanisms behind woody plant encroachment are poorly understood. Furthermore, woody plant encroachment has a marked effect on soil carbon as a result of the interactions between organic inputs and subsequent input mediated by soil microbes. However, whether affected ecosystems are a net source or sink of atmospheric CO<sub>2</sub> remains controversial. This study sought to understand the mechanisms that drive woody plant encroachment in grasslands as well as the biogeochemical consequences of woody plant encroachment on soil organic carbon and nitrogen pools.

There are four sections to this thesis:

(1) To elucidate the mechanisms that drive woody plant encroachment, I investigated how four encroaching shrub species (*Searsia erosa*, *S. burchellii*, *Diospyros lycioides* and *Eriocephalus ericoides*) in the semi-arid Middelburg area (Eastern Cape Province in South Africa) coexist and partition space under different grazing regimes (viz. continuous rest (no grazing), continuous grazing, summer grazing, and winter grazing). I used point-pattern analysis to assess the spatial ecology of these species. The spatial analysis showed that shrub density was significantly higher in the continuous and summer-grazing plots than in the winter-grazing and continuous-rest plots. The encroaching woody species were generally aggregated in most of the grazing plots. These findings indicate that (i) at a fine spatial scale,

1 grazing in the wet season promotes shrub encroachment; (ii) there is a tendency for  
2 aggregation of encroaching shrub species in the grazing plots.

3 (2) To elucidate the effects of woody plant encroachment on soil organic carbon  
4 (SOC) and soil total nitrogen (STN) storage, I measured the concentration of carbon (C) and  
5 nitrogen (N) and isotopic composition ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ ) in bulk soil in six pairs of adjacent  
6 grasslands (each replicated six times), in which encroached grasslands were invaded by  
7 woody species <100 years ago. The study sites were distributed across a precipitation gradient  
8 (300 to 1500 mm mean annual precipitation (MAP)). Soil samples were collected from four  
9 depths (0-10, 10-30, 30-60 and 60-100 cm) and analysed for SOC, STN,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ . I  
10 found a negative correlation between changes in soil organic C in the 0-100 cm soil layer and  
11 mean annual precipitation (MAP), as has been shown in North America. The most humid site  
12 (1500 mm MAP) had less C in shrub-encroached sites while the drier sites  
13 (300-350 mm MAP) had more C than their paired open grasslands. My findings also suggest  
14 that the bulk of SOC in the encroached grasslands was from  $\text{C}_4$  plants (grasses in our study  
15 sites). In the 0-10 cm soil layer at the 1500 mm site, SOC stocks under shrub encroachment  
16 were 26 % lower than in open grassland with a total loss of 20 % of bulk soil C at 0-100 cm  
17 depths due to shrub encroachment. At the intermediate-rainfall sites of 900, 700 and  
18 500 mm MAP, the bulk soil C in encroached sites and open grasslands in the uppermost soil  
19 layer were similar. Soil total nitrogen generally increased across all encroached sites  
20 compared to open grasslands, likely due to the presence of nitrogen-fixing plants.

21 (3) Soil organic carbon and nitrogen in the soil fractions at the 900, 700 and  
22 500 mm MAP sites were greater in shrub-encroached than open grasslands. In the soil  
23 physical fractions, the largest changes in SOC, ranging from 2.64 – 464 %, were in the coarse  
24 particulate organic matter (cPOM) and was lowest in the silt and clay fractions (6-32 %).

(4) In the same six pairs of encroached and unencroached grasslands across the  
aforementioned precipitation gradient, I measured soil microbial biomass, soil respiration,  
microbial species diversity and microbial functional diversity. Soil microbial biomass and soil  
respiration increased across all sites due to shrub encroachment. There was also a difference  
in microbial functional diversity between the encroached and adjacent open grasslands, which  
was most evident in the semi-arid regions. The increase in the microbial parameters in the  
semi-arid regions may be due to the increase of organic matter inputs in the encroached soils.

The study on the effects of woody plant encroachment on C and N storage indicated  
that (i) shrub-encroached grasslands potentially sequester more carbon in soils of semi-arid  
regions than in humid regions, (ii) SOC derived from C<sub>4</sub> grasses contributes over 50% of  
carbon in the encroached grasslands, (iii) changes in SOC and N due to shrub encroachment  
are mainly driven by gains in the coarse particulate organic matter (cPOM), and (iv) woody  
plant encroachment increases soil microbial biomass, soil respiration, microbial species  
diversity and alters functional diversity mostly in semi-arid regions.



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## CHAPTER 1: INTRODUCTION

### **Rationale for the research**

Tree and shrub densities have increased in many areas of southern Africa by 30-50% (Vegten 1984, Skarpe 1990, Hudak and Wessman 2001, Ward 2005, Kraaij and Ward 2006, Britz and Ward 2007). In South Africa, woody plant encroachment affects 10-20 million ha, seriously reducing the productivity for a country where >70% of its agricultural area is grazing lands only (Hoffman and Ashwell 2001). The mechanisms behind woody plant encroachment are poorly understood (Ward 2005), although heavy grazing, fire intensity and frequency, soil moisture, nutrients and global climate change have been attributed to grassland invasion by woody species (Ward 2005, Bond 2008, Van Auken 2009).

Some studies (Archer et al. 1995, Sankaran et al. 2008, Van Auken 2009) have identified heavy grazing by livestock as the major driver of woody species encroachment. Heavy grazing decreases the biomass and vigour of the grasses, thereby reducing the absorption of moisture from the upper soil layers (Knoop and Walker 1985). Ultimately, the competitiveness of grasses against establishing tree seedlings is reduced (Riginos et al. 2009, Ward et al. 2013). Heavy grazing also reduces grass fuel, which diminishes the probability and intensity of fire events and increases the frequency of juvenile trees and shrubs growing into taller, fire-resistant stages (Trollope 1980).

The effects of fire are analogous to those of a generalist herbivore, consuming both grassy and woody biomass (Bond and Keeley 2005). Without fire as a disturbance, open savannas can develop into woodlands and dense evergreen thickets (Gignoux et al. 2009). Formation of these dense thickets in grasslands reduces available fuel (in the form of grassy biomass) by shading out the herbaceous layer (Scholes et al. 1996). This reduction of the

herbaceous layer may limit the frequency and intensity of fires. Suppression of regular fire frequency increases woody plant density, by enabling shrub seedlings and saplings to develop to fire-resistant stages (Trollope 1980). Fire intensity is reputed to have little effect on the grass sward but influences the effects of fire on woody plants (Tainton 1999, Walters et al. 2004, Govender et al. 2006). Fire intensity is positively related to the mortality of woody plants (Trollope and Tainton 1986). However, the mortality of woody plants due to fire alone is reportedly low (Trollope and Tainton 1986, Midgley et al. 2010).

Woody plant encroachment has also been attributed to increased atmospheric carbon dioxide (Kgope et al. 2010, Ward 2010, Buitenwerf et al. 2012). Woody plants have a C<sub>3</sub> photosynthetic pathway, which is less efficient (lower net photosynthetic rate) at current atmospheric CO<sub>2</sub> levels than the C<sub>4</sub> pathway used by most southern African savanna grasses (Wolfe and Erickson, 1993). However, at higher atmospheric CO<sub>2</sub> levels than currently experienced, C<sub>3</sub> plants will have a higher net photosynthetic rate than C<sub>4</sub> plants (Wolfe and Erickson, 1993). As a result, the yield of C<sub>3</sub> plants will increase by 20-35% with a doubling of atmospheric CO<sub>2</sub>, while C<sub>4</sub> plants such as southern African grasses should only experience a 10% increase in yield (Wolfe and Erickson 1993).

### **Woody vegetation and carbon sequestration**

The increase of woody vegetation into deserts, grasslands and savannas is generally thought to have an impact on the global carbon balance (Schlesinger et al. 1990, Hibbard et al. 2001, Barger et al. 2011). The global concentration of carbon dioxide (CO<sub>2</sub>) in the atmosphere increased from 285 ppm at the end of the 19<sup>th</sup> century to about 366 ppm in 1998 and is currently at 402.8 ppm due to anthropogenic emissions of about 405 ± 60 gigatonnes of carbon (IPCC 2014). CO<sub>2</sub> is currently increasing at a rate of 1.5 ppm/yr or 3.3 Pg/yr

(1 Pg = petagram = 1 billion metric tons =  $1 \times 10^{15}$  g) (Vågen et al. 2005). The increase in atmospheric CO<sub>2</sub> and other greenhouse gases has led to an increase in the average global surface temperature of 0.6 °C since the late 19<sup>th</sup> century (Folland et al. 2001). The increase in atmospheric CO<sub>2</sub> contributes to this increase in atmospheric temperature (IPCC 2014). The major contributors of atmospheric CO<sub>2</sub> increase include emissions from fossil fuels, land-use change and soil degradation (IPCC 2014). Half of the CO<sub>2</sub> emitted accumulates in the atmosphere while the other half is sequestered by the oceans and the land, acting as carbon sinks. Understanding where, why and how these sinks occur is essential for managing biogeochemical cycles.

One of the most poorly understood carbon sinks is that of shrub-encroached grasslands (Goodale and Davidson 2002). A meta-analysis on woody plants showed that total biomass increased significantly at about twice ambient CO<sub>2</sub> (Curtis and Wang 1998). Given the extensive nature of woody plant expansion into grasslands both in southern Africa and beyond, and the expected increase in growth rates of C<sub>3</sub> plants, carbon sequestration may significantly increase the terrestrial carbon sink (Norris et al. 2001, Pacala et al. 2001). On the other hand, Goodale and Davidson (2002) argued that new measurements cast doubts on the increase of carbon storage due to woody plant encroachment. Jackson et al. (2002) showed that there are smaller increases and in some cases reductions of carbon stocks in shrub-invaded grasslands. These findings require that more research be carried out so as to understand how these carbon sinks function.

In this study, I investigated the effects of woody plant invasion along a precipitation gradient (mean values ranging from 300 to 1,500 mm per yr) by comparing carbon and nitrogen budgets in grasslands. I used a four-way approach to determine the importance of woody plant encroachment for ecosystem carbon storage, viz. (1) spatial pattern analysis of



encroaching species; (2) direct C and N measurement in three soil physical fractions; (3) assessment of carbon and nitrogen isotopes in plants; and (4) determination of soil microbial biomass and microbial functional diversity.

#### **Spatial pattern of encroaching species**

Spatial pattern analysis is a tool that can be used to further clarify the mechanisms of woody plant encroachment. Spatial point-pattern analysis compares the distribution of trees (as points) with a distribution in a null model, showing aggregation or regularity where it differs from a null model and randomness where distribution resembles that of a null model (Wiegand and Moloney 2004, Pillay and Ward 2012). Studying community spatial patterns allows ecologists to determine the underlying processes for the observed pattern (Wiegand and Moloney 2004, Jacquemyn et al. 2010, Pillay and Ward 2012, Wiegand et al. 2013), which may elucidate intraspecific and interspecific interactions such as competition and facilitation (Wiegand and Moloney 2004, Wiegand et al. 2013). Intense competition among woody species can result in a regular pattern of the shrubs while facilitation may give rise to a clustered pattern of the shrubs causing woody plant encroachment (Jeltsch et al. 2000, Wiegand et al. 2006, Meyer et al. 2008). Determination of the spatial pattern of encroaching shrubs may further help in elucidating the processes of shrub encroachment.

## **Storage of carbon and nitrogen in soil physical fractions**

One challenge to understanding the effects of shrub encroachment on soil organic carbon (SOC) and nitrogen (N) is that the majority of studies in shrub-encroached grasslands have focused on bulk soil C and N rather than C and N contained in different soil fractions (Throop et al. 2013). Soil is comprised of a continuum of fractions that contain C that differ widely in turnover rates (Jenkinson and Rayner 1977, Paul and Juma 1981, Baisden et al. 2002). SOC fractions can be physically separated into: (1) coarse particulate organic matter (cPOM), a labile fraction with a mean residence times (MRT's)  $\approx$  1-2 years, (2) microaggregate, protected organic matter ( $M_{agg}$ ), a slow fraction with a MRT's  $\approx$  25 years and (3) silt and clay fraction, a passive, recalcitrant fraction with a MRT  $\approx$  100-1000 years (Parton et al. 1987, Jenkinson et al. 1990). These physical fractions may capture the effects of woody plant encroachment on soil C and N accumulation better than unfractionated soils (Throop et al. 2013).

## **Carbon and nitrogen isotopes**

Woody plant encroachment in grasslands affords a unique opportunity to use stable isotopes to study soil organic matter dynamics following this shift in vegetation structure. The broad isotopic differences between woody ( $C_3$ ) and grass (all  $C_4$  plants in our study sites) community types allows for isotopic tracing of SOC accumulation following woody plant encroachment. In particular, carbon isotopes may show whether SOC accumulations are the result of increases in the most recent organic-matter inputs derived from woody vegetation or from retention of older organic C derived from the previous grassland, or from a mixture of both sources (Boutton and Liao 2010).

The natural abundance of  $\delta^{15}\text{N}$  in plants and soils may provide information on N sources and transformations in ecosystems (Michelsen et al. 1996, Boutton and Liao 2010) and is considered an integrator of soil N-cycling processes as affected by both the biotic and abiotic environment (Robinson 2001, Dawson et al. 2002). Multiple factors influence plant and soil  $\delta^{15}\text{N}$  values such as temperature, precipitation, quantity and quality of litter inputs, soil nitrogen sources, and isotopic fractionation resulting from N transformations (Piccolo et al. 1994, Amundson et al. 2003). However, the influences of woodland development on N-cycling should be apparent in the natural abundance of  $^{15}\text{N}$  in the plant-soil system (Boutton and Liao 2010).

#### **Soil microbial parameters**

Many studies on woody plant encroachment focus on soil chemical and physical properties (Ross et al. 1999, Chen et al. 2000, Zeng et al. 2009). However, approaches based on the characterization of active soil organic-matter pools with more rapid turnover rates have been suggested as more effective measures of monitoring soil changes due to land-cover change (Sparling 1992, Liao and Boutton 2008). Turnover times of soil physical and chemical parameters are comparatively slow, from tens to thousands of years, relative to the turnover times measured in days for soil microbial parameters (Dalal 1998).

Changes in soil microbial biomass have been proposed as an early indicator of the effects of land-cover change before the effects are detectable in soil chemical and physical parameters (Powlson et al. 1987, Liao and Boutton 2008). The microbial quotient (ratio of soil microbial biomass to total SOC ( $C_{\text{mic}} / C_{\text{org}}$ ) has been used as a measure of C availability to decomposer organisms and/or the quality of the SOC (Sparling 1992). Quantification of the microbial respiration quotient ( $q\text{CO}_2$ ) reflects the ability of soil microbial biomass to

convert available substrate to biomass and is an indicator of microbial efficiency (Bradley and Fyles 1995, Wardle and Ghani 1995).

### **Aims and Objectives of the study**

This study sought to understand the mechanisms that drive woody plant encroachment of grasslands and the biogeochemical consequences of woody plant encroachment on soil organic carbon and nitrogen pools across a precipitation gradient (300 mm to 1500 mm, MAP) in South Africa.

The specific objectives of the study were:

(1) To determine the spatial patterns of encroaching shrub species under different grazing regimes in a semi-arid savanna,

(2) Determine the relative importance of C derived from woody vegetation ( $C_3$ ) versus grassland vegetation ( $C_4$ ) using the natural isotopic difference between  $C_4$  grasses and  $C_3$  woody plants,

(3) Quantify SOC and N sequestration in soil fractions following conversion from grassland to woodland across a precipitation gradient, and

(4) Establish microbial functional diversity following grassland-to-woodland succession.

### **Study sites**

The study was carried out in South Africa at six study sites along a precipitation gradient (Fig. 1). Mean annual rainfall ranged from 1500 mm to 300 mm. The six sites are KwaMbonambi (1500 mm MAP), Stanger (900 mm MAP), Bergville (700 mm MAP), Bloemfontein (500 mm MAP), Pniel (350 mm MAP) and Middelburg (300 mm MAP) (Table 1).

## Thesis structure

**Chapter 1** is a brief introduction of the thesis that outlines the key questions, objectives, study sites, and structure of the thesis. **Chapter 2** presents a field study on the effects of different grazing regimes on woody plant encroachment and the spatial pattern analysis of the encroaching species in a semi-arid savanna of South Africa. The study demonstrates the importance of grazing in the wet season as one of the major drivers of shrub encroachment. The study also suggest that encroaching woody plants are mostly clustered around each other (i.e. that there is facilitation). **Chapter 3** reports on a field study on the relative importance of C and N derived from woody plant species vs. grassland vegetation using carbon and nitrogen isotopes. The study showed a negative correlation in changes in SOC between encroached and open grassland with increasing mean annual precipitation. The study also indicated that the bulk of SOC in the encroached areas is from grasses. In **Chapter 4**, I present a field study on the effects of woody plant encroachment on C and N pools in different soil physical fractions. The study consists of determining the importance of soil physical fractions in determining the changes in soil C and N due to shrub encroachment. **Chapter 5** presents a field study on the effects of shrub encroachment on soil microbial properties such as soil respiration, soil microbial biomass and microbial functional diversity. The chapter emphasises that microbial properties significantly change in semi-arid regions while in humid regions there are no significant changes. Finally, **Chapter 6** presents general conclusions based on the experimental results obtained over the duration of the entire project. New avenues for future research are also proposed in this final chapter.

All chapters in the thesis, with the exception of the Introduction (**Chapter 1**) and the Conclusions (**Chapter 6**), are formatted according to the style of a particular targeted journal as indicated on the title page of that chapter. As a result, some repetition and inconsistency of format is unavoidable. The chapters and their targeted journals are as follows:

**Chapter 2:** “Spatial patterns of encroaching shrub species under different grazing regimes in a semi-arid savanna (Eastern Karoo, South Africa)” has been provisionally accepted in the *African Journal of Range and Forage Science*.

**Chapter 3:** “Woody plant encroachment enhances carbon sequestration in semi-arid regions and decreases sequestration in humid regions of South Africa”. Formatted for *Nature*.

**Chapter 4:** “Storage of carbon and nitrogen in soil physical fractions following woody plant invasion of grassland”. Formatted for *Plant and Soil*.

**Chapter 5:** “Soil microbial biomass and functional diversity in shrub-encroached grasslands along rainfall gradient”. Formatted for *Soil Biology and Biogeochemistry*.

Table 1.1 Study site description according to Mucina and Rutherford (2010)

Site	GPS coordinates	Annual Rainfall (mm)	Temperature (°C) Min      Max		Biome	Soil Characteristics	Major plant species
KwaMbonambi	28° 49' 60.61"S 32° 16' 96.92"E	1500	3.5	35	Maputaland wooded grasslands	Quaternary redistributed sands supporting yellowish redistributed sands of the Berea formation	<i>Sporobolus fimbriatus</i> , <i>Digitaria natalensis</i> (grasses); <i>Diospyros lycioides</i> (shrub); <i>Terminalia sericea</i> (tree)
Stanger	29° 18' 59.05" S 31° 22' 13.24"E	900	5.8	32.6	KwaZulu-Natal Coastal Belt	Ordovician Natal group sandstone	<i>Themeda triandra</i> , <i>Aristida junciformis</i> (grass species); <i>A. karroo</i> (tree)
Bergville	28° 79' 06.30" S 29° 38' 98.40"E	700	5.8	32.6	KwaZulu-Natal moist grasslands	Ordovician Natal group sandstone	<i>Themeda triandra</i> , <i>Hyparrhenia hirta</i> (grasses); <i>Acacia karroo</i> , <i>A sieberiana</i> (trees)
Bloemfontein	28° 59' 16.77"S 26° 16' 54.24E	450	0	32	Bloemfontein dry grasslands	Sedimentary mudstones and layers of sandstone	<i>Aristida congesta</i> , <i>A. diffusa</i> , <i>Cynodon dactylon</i> (grass species); <i>Acacia karroo</i> (tree)
Pniel	28° 34' 50.00"S 24° 30' 30.70"E	350	-4.1	37.5	Kimberley thornveld	Sandy to loam soils of the Hutton soil form	<i>Eragrotis curvula</i> , <i>Schmidtia pappophoroides</i> (grasses); <i>Acacia erioloba</i> , <i>A tortilis</i> , <i>A. karroo</i> , <i>A. mellifera</i> , <i>Tarchonanthus camphoratus</i> (trees)
Middelburg	31° 25' 98.83"S 24° 58' 82.10"E	300	-7.2	36.1	Eastern Upper Karoo	Sandy to loam soils of the Hutton soil form	<i>Aristida</i> and <i>Eragrostis</i> (grasses); <i>Searsia erosa</i> , <i>S. burchellii</i> , <i>Diospyros lycioides</i> and <i>Eriocephalus ericoides</i> (shrubs)

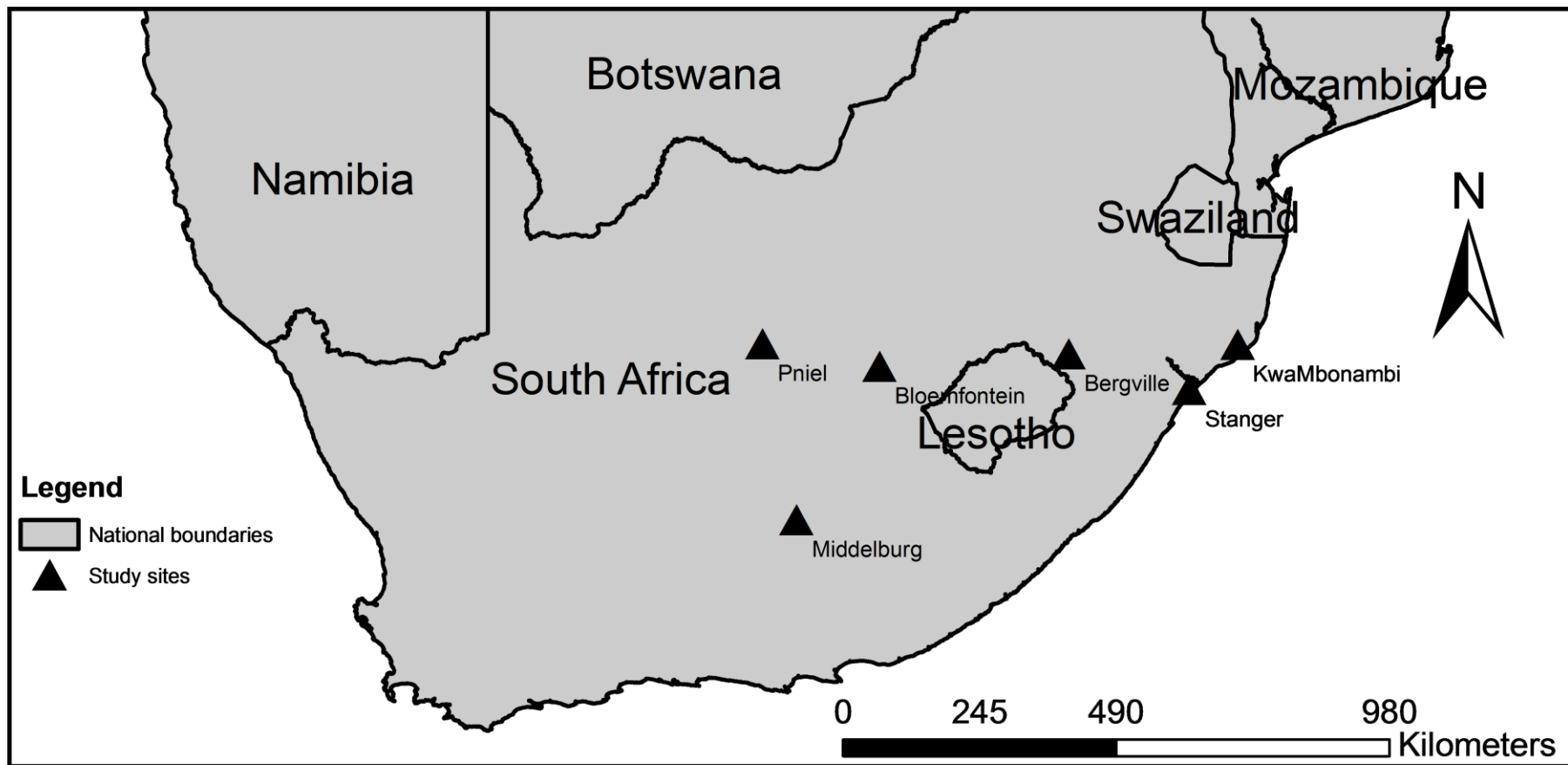


Fig. 1: Study sites relative to major cities of South Africa.



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**CHAPTER 2: SPATIAL PATTERNS OF ENCROACHING SHRUB SPECIES  
UNDER DIFFERENT GRAZING REGIMES IN A SEMI-ARID SAVANNA  
(EASTERN KAROO, SOUTH AFRICA)**

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preparation.



## ABSTRACT

Woody plant encroachment is increasing in arid and semi-arid regions of the world. We investigated the structure and fine-scale spatial pattern of encroaching species and how these patterns vary with different grazing regimes in semi-arid regions. In this study, we investigated how four encroaching shrub species (*Searsia erosa*, *S. burchellii*, *Diospyros lycioides* and *Erioccephalus ericoides*) in Middelburg (Eastern Cape, South Africa) coexist and partition space under different grazing regimes (viz. continuous rest, continuous, summer and winter grazing). We used point-pattern analysis to assess the spatial ecology of these species. We also used an index of integration (*mingling index*), where low values indicate that they are surrounded by conspecifics and high values indicate that they are surrounded by heterospecifics. The three shrub species were generally surrounded by heterospecifics except in the winter plot where *S. burchellii* and *D. lycioides* were surrounded by conspecifics. We found that the shrub species were generally aggregated in most of the grazing plots. These findings indicate that (1) at a fine spatial scale, grazing in the wet season promotes shrub encroachment, and (2) there is a tendency to aggregation among encroaching shrub species in the grazing plots.

## Keywords

Spatial pattern; encroaching shrubs; grazing regimes; competition; facilitation; point pattern analysis

## 1   **Introduction**

2  
3   Woody plant density is increasing globally in many arid and semi-arid regions (Archer et al.  
4   1995, Springsteen et al. 2010) but our understanding of the mechanisms driving this process  
5   is limited (Archer et al. 1995, Ward 2005). However some progress is being made in  
6   understanding the mechanisms behind bush encroachment (O'Connor et al. 2014, Ward et al.  
7   2014). Grassland invasion by woody species have been attributed to factors such as heavy  
8   grazing, fire intensity and frequency, soil moisture, nutrients and global climate change  
9   (Ward 2005, Bond 2008, Van Auken 2009). Regular fire frequency suppresses woody plant  
10   growth, destroying adult shrubs, shrub seedlings and saplings, preventing their development  
11   to fire-resistant stages. Woody plant encroachment has also been attributed to increased  
12   atmospheric carbon dioxide (Kgope et al. 2010, Ward 2010) due to the greater net  
13   photosynthetic efficiency of woody C<sub>3</sub> plants than competitor C<sub>4</sub> grasses that are often  
14   dominant in hot environments (Wolfe and Erickson 1993). Some studies (Archer et al. 1995,  
15   Sankaran et al. 2008, Van Auken 2009) have identified heavy grazing by livestock as the  
16   major driver of woody species encroachment. Heavy grazing decreases the biomass and  
17   vigour of the grass, reducing the moisture absorption from the upper soil layers (Knoop and  
18   Walker 1985). Ultimately, the competitiveness of the grass against establishing tree seedlings  
19   is reduced (Riginos et al. 2009, Kgosikoma et al. 2012, Ward et al. 2013). Heavy grazing also  
20   reduces grass fuel, which diminishes the probability and intensity of fire events and increases  
21   the frequency of juvenile trees and shrubs growing into taller, fire-resistant stages (Trollope  
22   1980). Thus, heavy grazing may alter plant composition and structure of encroaching  
23   rangelands.

24           Composition of any mature vegetation stand, such as grassland, is shaped by  
25   competition (Clements et al. 1929, Wiegand et al. 2008) although there is increasing evidence

of facilitation among plant species (Callaway and Walker 1997, Jeltsch et al. 2000, Bertness and Ewanchuk 2002, Bruno et al. 2003). Intense competition among woody species can result in a regular pattern of the shrubs while weak competition from grasses may give rise to a clustered pattern of the shrubs causing woody plant encroachment (Jeltsch et al. 2000, Wiegand et al. 2006, Meyer et al. 2008). Facilitation among woody species can also cause woody plant encroachment. Facilitation can be due to seed dispersal, nurse-plant syndrome and environmental heterogeneity. A facilitative nurse-plant syndrome often refers to seedling establishment under canopies of adult trees. In such interactions, the seedlings profit from protection against harsh temperatures, higher available soil moisture and nutrients, and reduced soil compaction and erosion (Flores and Jurado 2003). The nurse-plant syndrome has mainly been reported in arid and semi-arid regions (Tielbörger and Kadmon 2000, Chen et al. 2011, Schleicher et al. 2011).

Assessing vegetation structure and spatial patterns may reveal inter- and intra-specific interactions such as competition and facilitation (Dale 1999, Getzin et al. 2006). Spatial pattern analysis is useful in detecting competition or facilitation by assessing the spatial distribution of trees and determining the scale at which the spatial pattern is significantly aggregated or regular (Wiegand and Moloney 2004, Getzin et al. 2006, Punchi-Manage et al. 2013). There are two major indirect means of determining spatial distribution of plants, namely point-pattern and nearest-neighbour analysis (Shackleton 2002, Wiegand and Moloney 2004). In point-pattern analyses, the position of a plant in a plot is represented by a point, and the spatial pattern analysis indicates whether the distribution of the points is random, aggregated or regular by comparing the distribution with the pattern of the plants under a specific null model (Wiegand and Moloney 2004). However, spatial pattern analyses cannot detect competitive interactions when competition does not result in differential mortality of individual plants (Getzin et al. 2006). Nearest-neighbour analysis, on the other

hand, may provide a useful tool for detecting subtle interactions, where competition may result in reduced growth rather than mortality (Shackleton 2002, Getzin et al. 2006). Nearest-neighbour analysis shows that if competition is present there will be a substantial decrease in size of one or more competing neighbours (Shackleton 2002). This analysis works on the premise that there is a positive correlation between a size index (usually canopy diameter) and distance between competing neighbours (Shackleton 2002). It is therefore expected that large shrubs should have smaller neighbours. Contrastingly, facilitation among shrubs may result in increased growth, resulting in a negative correlation (Schleicher et al. 2011).

This study sought to understand the effects of grazing regime on encroachment by shrub species and their spatial patterns in the different grazing regime plots. We employed spatial indices, spatial point-pattern analyses and nearest-neighbour distances to quantify the structures and spatial patterns of encroaching semi-arid shrubs. Our predictions were: (1) Grazing in the wet season increases the density of encroaching species because reduction of grass allows water to percolate into the sub-soil, thereby allowing woody species to proliferate (Cipriotti and Aguiar 2012, Ward et al. 2013); (2) The spatial pattern of shrub species should be regular in all grazing regimes due to competition (Shackleton 2002).

## **Methods**

### **Study site**

The study was conducted at the Grootfontein Research Centre, Middelburg in the Eastern Cape (28° 35' 0" S, 24° 26' 0" E). Middelburg receives an annual rainfall of 300 mm, with most rainfall occurring in autumn, with the highest rainfall (63.8 mm) in March (summer) and the lowest (10.1 mm) in June (winter) (Du Toit 2010). The temperatures range from

-7.2 °C (July) to 36.1 °C (January) (Mucina and Rutherford 2010). The soils are mainly aeolian sands and andesite clays. The vegetation in the vicinity of Middelburg falls under the Nama-Karoo biome (Mucina and Rutherford 2010). The principal shrubs are *Pentzia*, *Eriocephalus*, *Lycium* and *Searsia* species. The dominant grasses include *Aristida*, *Digitaria* and *Stipagrostis* species. Grasses tend to be more common in depressions and on sandy soils.

## Field study

The Bergkamp grazing trial plots were established in 1943 at Grootfontein Agricultural College in Middelburg, Eastern Cape (South Africa). The purpose of these plots was to monitor the effects of sheep grazing on species composition. At the time of establishment, area covered by shrubs was less than 5%. Grazing intensity in the trial plots was 2 sheep / ha (through out the research period) which is in line with the recommended stocking rates for the Nama-Karoo biome. The plots had continuous grazing (10.4 ha), summer grazing (1.7 ha), winter grazing (1.7 ha), and continuous rest (0.5 ha) treatments. There was no replication when these plots were established.

There has been a noticeable increase in shrub encroachment in the grazed plots compared to the continuous-rest plot since the beginning of the study. The encroaching shrub species in the grazing plots are *Searsia erosa*, *S. burchellii*, *Diospyros lycioides* and *Eriocephalus ericoides*. *Eriocephalus ericoides* was the major encroaching species in the continuous-rest plot while species had a low density in other grazing plots.

In this study, individual grazing plots (Figure 1 and 2) were divided into sub-plots. The sub-plots were treated as replicates. We recognize this as pseudoreplication but believe the patterns are important enough to be demonstrated. Two sub-plots were laid out in each of the three grazing treatments. In the continuous rest treatment, only one sub-plot was established because encroachment was limited to a small portion of the plot only; we wished

to ascertain the interactions among the woody species. The dimensions of the sub-plots were determined by the density of the woody species. We aimed to have more than 40 individuals in each plot, where shrub density was very low we used bigger plots in order to have the minimum required individuals for spatial analysis. In the summer grazing plot (Figure 1 and 2), the sub-plots measured 40 m by 40 m. In the winter grazing treatment, the sub-plots measured 50 m by 50 m. In the continuous grazing and continuous rest treatment, the plots were 25 m by 25 m (in the continuous rest plot, only a small portion measuring approximately 25 m by 25 m was encroached). Each sub-plot was laid out as a Cartesian plane with the origin (0,0) as the reference point for the location of each shrub. The x- and y-coordinates of each encroaching shrub (*Searsia erosa*, *S. burchellii*, *Diospyros lycioides* and *Eriocephalus ericoides*) were determined using a tape measure. Data on morphological structure (specifically, shrub height and crown diameter) of each plant were also recorded.

### **Stand structure and composition analysis**

Differences in stand structure (specifically, shrub height and crown diameter) among species in the different plots were analysed using analysis of variance (ANOVA). The mean nearest distance among individuals in each sub-plot were calculated. The Von Gadow mingling index ( $M_i$ ) (Chen et al. 2011) was calculated to determine the relative spatial mix of each species. The Von Gadow mingling index is the proportion of the  $n$  nearest neighbours that do not belong to the same species.  $M_i$  for sample tree  $i$  is determined by Graz (2004) as:

$$M_i = \frac{1}{n} \sum_{j=1}^n m_j \quad (1)$$

where  $n$  is the number of neighbours considered and  $i$  denotes the sample tree evaluated.  $m_j = 1$  if the shrub is of another species than  $i$ , otherwise it is 0. The arithmetic mean,  $M_{sp}$  of the

observed values of  $M_i$  for a species  $sp$ , is such that  $0 \leq M_{sp} \leq 1$ . A value of  $M_{sp}$  close to zero implies that the individuals of that species occur in groups, thus indicating a low level of species mingling. High values of  $M_{sp}$  close to one, on the other hand, indicate that the individuals of the species occur in isolation from other individuals of the same species.

The  $M_{sp}$ , however, does not take into consideration the proportion of each species within the plot. Equation 2 therefore was used to evaluate the  $M_{sp}$  in relation to the proportion of the species within the grazing sub-plots (Graz 2004).

$$M = \frac{P_{sp}}{1 - M_{sp}} \quad (2)$$

where  $M$  is the standardised mingling index for a species in a plot,  $P_{sp}$  is the proportion of number individuals of a species in relation to the total number of individuals of all species in a plot and  $M_{sp}$  is the arithmetic mean of the observed values of  $M_i$  for a species  $sp$ .

We also used the size differentiation index (height and crown differentiation), which is the proportion of the  $n$  nearest neighbours of a given reference shrub that are bigger than the reference shrub. It is determined as indicated in equation 3:

$$U_i = \frac{1}{n} \sum_{j=1}^n V_j \quad (3)$$

where  $U$  is the size differential index,  $n$  is the number of neighbours considered and  $i$  denotes the sample shrub evaluated.  $v_j$  is 1 if the shrub is bigger than  $i$ , otherwise it is 0 (Graz 2004). For the determination of both the Von Gadow mingling and size differentiation indices, we used four nearest neighbours for each individual shrub as recommended by Shackleton (2002) and Pillay and Ward (2012). The xy coordinates and height/crown diameter were used

to determine the height/crown differentiation indices and for the nearest-neighbour analysis we used xy coordinates and the crown diameters.

### Spatial pattern analysis

The unbiased K-function univariate estimator for detecting intraspecific interactions is:

$$K(r) = n^{-2} |A| \sum_{i=1}^n \sum_{i \neq j} w_{ij}^{-1} I_r(u_{ij}) \quad (4)$$

where  $K$  is the Ripley univariate function,  $n$  is the number of shrubs in the study area  $A$ .  $u_{ij}$  is the distance between the  $i^{th}$  (focal) shrub and the  $j^{th}$  (neighbouring) shrub, where the focal shrub tree is located within area  $A$ .  $I(u_{ij})$  is an indicator function, equivalent to 1 if  $u_{ij} < r$  and is otherwise = 0. The term  $w_{ij}$  corrects for the edge effect, where  $w_{ij}$  is equal to 1 if the entire circumference of the circle lies within  $A$  (Gray and He 2009). However, instead of using the  $K$  function, the square-root transformation of the  $K(r)$  function, called the  $L$  function, is used (Wiegand and Moloney 2004). This  $L$  function is easily interpreted because it removes the scale dependence of  $K(r)$  and stabilises the variance (Wiegand and Moloney 2004). The  $L$ -function that was used to compare the univariate spatial pattern of dominant species in each grazing plot was determined as:

$$L(r) = \sqrt{\frac{K(r)}{\pi}} \quad (5)$$

where  $L(r) = 0$  for a random pattern,  $L(r) < 0$  for a regular pattern and  $L(r) > 0$  for a clustered pattern.  $K(r)$  is as in equation 4 above. The bivariate  $K$ - function ( $K_{12}(r)$ ) is the expected number of points of pattern 2 within a given distance  $r$  from an arbitrary point of pattern 1 divided by the intensity of pattern 2 points (Wiegand and Moloney 2004). The second-order bivariate estimate was defined as follows:

$$K_{12}(r) = (n_1 n_2)^{-1} |A| \sum_{i=1}^{n_1} \sum_{j=1}^{n_2} w_i^j I_r(u_{ij}) \quad (6)$$



where  $n_1$  and  $n_2$  are populations from species 1 and 2 respectively in area  $A$ . Other terms are interpreted as in equation 4 above. The  $L_{12}(r)$  function was determined as follows:

$$L_{12}(r) = \sqrt{\frac{K_{12}(r)}{\pi}} - r \quad (7)$$

If patterns 1 and 2 are randomly associated,  $L_{12}(r) = 0$ . When there is facilitation,  $L_{12}(r) > 0$ .

If the shrubs are competing,  $L_{12}(r) < 0$  (Gray and He 2009).  $K_{12}(r)$  is calculated as in equation 6 above.

The O-ring statistic of a univariate point pattern counts the number of points in a ring around an arbitrarily chosen point at a specific distance  $r$ . This is done for all points in the pattern. The bivariate O-ring statistic can be defined as follows:

$$O_{12}(r) = \lambda_2 g_{12}(r) \quad (8)$$

where  $g_{12}(r) = \frac{dK_{12}(r)}{dr} / 2\pi r$  in which  $K_{12}(r)$  is as in equation 5 above (Wiegand and Moloney 2004).

The confidence envelopes for  $L$  and O-ring statistics were estimated from 999 Monte Carlo simulations using the complete spatial randomness null model for univariate analysis and the toroidal shift null model for the bivariate analysis (Wiegand and Moloney 2004). The confidence envelopes were estimated using the 5<sup>th</sup> lowest and the 5<sup>th</sup> highest value for each distance  $r$ . In the bivariate case, if the  $L(r)$  and O-ring statistic exceeds the upper confidence limit, it indicates significant aggregation at the  $r$  spatial scales where the deviation occurs. If the function is below the lower confidence limit, it indicates significant spatial repulsion (i.e. competition). When the function lies within the confidence limits, the distribution is considered random. To avoid the problems of Type I errors associated with multiple testing (Loosemore and Ford 2006, Perry et al. 2006), we conducted a goodness-of-fit (GoF) test for each analysis. Spatial analysis and GoF test were done using Programita software (Wiegand and Moloney 2004).

## Nearest-neighbour analysis

We used nearest-neighbour analysis to infer fine-scale competition. Competition does not always lead to significant mortality and may only result in growth reduction in one or more neighbouring plants (Dale 1999, Pillay and Ward 2012). We determined the correlation between the sum of canopy diameters of four nearest neighbours plus the canopy diameter of the focal shrub and the sum of the distance from the four nearest neighbours to the focal shrub (Pillay and Ward 2012). If the shrubs are directly competing, then the nearest-neighbour distance should be smaller for smaller shrubs than for large shrubs. A significant positive correlation between nearest-neighbour distance and shrub size indicates competition and a significant negative correlation indicates facilitation. The data points were not independent, so the nearest-neighbour correlation analyses were done using a Mantel test (Legendre and Fortin 2010).

## Results

The continuous rest plot and the winter grazing sub-plots had a low shrub density of 60 stems/ha and 156 stems/ha compared to continuous grazing sub-plots which had an average of 1 708 stems/ha and summer grazing sub-plots with an average of 1524 stems/ha (Table 1, Figure 3). *S. erosa* in the winter-grazing sub-plots had a larger crown diameter ( $3.69 \pm 0.14$  m,  $F_{2, 124} = 12.79$ ,  $P < 0.0001$ ) than *S. erosa* in continuous and summer grazing sub-plots ( $2.02 \pm 0.14$  and  $1.98 \pm 0.87$  respectively) (Table 2). *D. lycioides* had also a larger crown diameter in the winter sub-plots ( $2.39 \pm 0.11$  m,  $F_{2, 209} = 10.55$ ,  $P < 0.0001$ ) compared to continuous and summer grazing sub-plots ( $0.99 \pm 0.12$  and  $0.99 \pm 0.08$ ). *S. burchellii* was the only species that was significantly shorter in the winter sub-plots ( $1.4 \pm 0.56$  m,  $F_{2, 263} = 4.522$ ,  $P = 0.009$ ) compared to continuous and summer grazing sub-plots ( $1.61 \pm 0.07$  and  $1.735 \pm 0.16$ ). The standardised Von Gadow mingling index ( $M_{sp}$ ) indicated that the three

species are highly mingled ( $M_{sp} > 0.75$ ) except in the winter grazing sub-plots where *S. burchellii* and *D. lycioides* indicate that each of these species occurs in clusters ( $M_{sp} < 0.5$ ) (Table 2).

#### Univariate pattern analysis

In this study, both the K function and the O-ring statistic were used because in spatial analysis a combination of two or more tests is more informative (Chen et al. 2011). In continuous grazing sub-plot 1, the L(r) function showed that *S. burchellii* and *D. lycioides* were significantly aggregated up to 12 m (GoF test:  $p = 0.01$ ). However the O(r) function showed aggregation at 0-2.5 m and at 4-7 m for *S. burchellii* (GoF test:  $p = 0.01$  and  $P = 0.01$ , respectively) while this function indicated that *D. lycioides* was aggregated up to 8 m (GoF test:  $P = 0.01$ ). *D. lycioides* showed a random pattern as indicated by both L(r) and O(r) functions while *S. burchellii* and *S. erosa* showed aggregation up to 2 m and 1 m (GoF test:  $P = 0.01$  and  $P = 0.04$ , respectively), according to the L (r) function, in continuous-grazing sub-plot 2 (Table 3).

*D. lycioides* in summer-grazing sub-plot 2 was aggregated up to 8 m (GoF test:  $P = 0.01$ ) according to the L(r) function whereas the O(r) showed aggregation up to 2 m (GoF test:  $P = 0.03$ ). The L(r) function showed aggregation of all three species (i.e. except *E. eriocephalus*, which did not occur in these plots) at different scales in summer-grazing sub-plot 2 (Table 3). However, the O(r) function showed a random pattern at all scales for *S. erosa* and *D. lycioides* and a significant repulsion (i.e. competition) at 14-16 m (GoF test:  $P = 0.04$ ) for *S. burchellii*.

In winter-grazing sub-plot 1, L(r) indicated *S. erosa* aggregation at 2-4 m (GoF test:  $P = 0.03$ ). In winter-grazing sub-plot 2, the L(r) and O(r) showed significant aggregation for *S. erosa* at 0-8 m and 6-7 m (GoF test:  $P = 0.02$  and  $P = 0.04$ , respectively).

## Bivariate pattern analysis

In continuous-grazing sub-plot 1, the interaction between *S. burchellii* vs *S. erosa* and *D. lycioides* showed significant attraction at all scales (GoF test:  $P = 0.01$ ), according to the  $L_{12}(r)$  function, while the  $O_{12}(r)$  function showed aggregation up to 7 m (GoF test:  $P = 0.01$ ) (Table 4, Appendix 1). The *D. lycioides* vs *S. burchellii* + *S. erosa* interaction in continuous-grazing sub-plot 1 was significantly aggregated (GoF test:  $P = 0.01$ ) at all scales according to the  $L_{12}(r)$  function. The  $O_{12}(r)$  function showed significant aggregation up to 7 m (GoF test:  $P = 0.01$ ) (Table 4, Appendix 1). Interactions in the continuous-grazing sub-plot 2 were all random.

In summer-grazing sub-plot 1,  $L_{12}(r)$  showed some significant attraction between *S. erosa* vs *D. lycioides* and *S. burchellii* from 2-3 m (GoF test:  $P = 0.02$ ). However, the  $O_{12}(r)$  function showed that the pattern was mostly random. In summer-grazing sub-plot 2, *S. erosa* vs *D. lycioides* and *S. burchellii* showed significant attraction at 1-3 m (GoF test:  $P = 0.03$ ) according to the  $L_{12}(r)$  function but  $O_{12}(r)$  indicated that the pattern was random. In summer-grazing sub-plot 2, the  $L_{12}(r)$  function also showed a significant attraction between *S. burchellii* vs *D. lycioides* and *S. erosa* between 6-7 m (GoF test:  $P = 0.03$ ). The  $O_{12}(r)$  showed significant attraction between *D. lycioides* vs *S. erosa* and *S. burchellii* up to 3 m (GoF test:  $P = 0.04$ ).

In winter-grazing sub-plot 1, *S. erosa* vs *D. lycioides* and *S. burchellii* were significantly attracted between 2-6 m (GoF test:  $P = 0.03$ ) according to the  $L_{12}(r)$  function. The  $O_{12}(r)$  showed attraction at 2-3 m only (GoF test:  $P = 0.04$ ). In winter-grazing sub-plot 2, *S. erosa* vs *D. lycioides* and *S. burchellii* showed some significant repulsion (i.e. competition) between 7-25 m (GoF test:  $P = 0.03$ ) while the  $O_{12}(r)$  index showed a random distribution.

## Nearest-neighbour analysis

There was a significant positive correlation between sum of the distance to the nearest neighbour and the sum of the canopy diameters of the focal shrub and its neighbours in all the grazing plots for all the shrub species (Table 5). However, although the P-values were significant (because of the large number of data points), the relationships explained very little (<5 %) of the variation in all the grazing plots (Table 5).

## Discussion

During the establishment of our grazing trials in 1943, the grazing plots were not replicated. This lack of replication, unfortunately, constrained our ability to assess the effects of different grazing regimes on shrub encroachment. Nonetheless, our results showed that shrub density was high in continuous- and summer-grazing plots relative to winter-grazing and continuous-rest plots. This finding supported our prediction that the timing of grazing had an effect on shrub encroachment. High shrub encroachment in the summer- and continuous-grazing plots may indicate that water is a major limiting factor for both woody and grass species in semi-arid regions (Ward 2005). Grasses mainly use topsoil water whereas shrubs utilise sub-soil moisture (Knoop and Walker 1985, Ward et al. 2013). Removal of grass by sheep herbivory during the rainy season may allow water to percolate into the sub-surface areas, thereby allowing woody species to proliferate (Cipriotti and Aguiar 2012, Ward et al. 2013). High shrub density in continuous- and summer-grazing plots indicates that grass removal in the wet season (in continuous grazing and summer grazing plots) affords shrub seedlings an opportunity to germinate with less competition from established herbaceous plants (Bond 2008, Ward et al. 2013). Conversely, there was relatively little encroachment in the (dry) winter-grazed plot, presumably because, when the aboveground grass material is dead, there

1 is insufficient soil moisture for shrub seeds to germinate. A very low density of encroaching  
2 species was conspicuous in the continuous-rest plot, further emphasising the importance of  
3 grazing on shrub encroachment. The significant difference in species density in the grazing  
4 plot may suggest that increased atmospheric carbon dioxide is not a major driver of woody-  
5 plant encroachment, contrary to suggestions by Kgope et.al (2010) and Ward (2010). Further  
6 experimental studies would be needed to establish whether these mechanisms are indeed  
7 functional.

8         *S. burchellii* and *D. lycioides* had very high densities in the continuous- and summer-  
9 grazing plot relative to the winter-grazing plot. These two species were ten times more dense  
10 in the continuous- and summer-grazing than in the winter-grazing plots (Table 1). The  
11 presence of *S. erosa* in the winter-grazing plot at high density may indicate that this shrub  
12 species is highly competitive against grass and may be facilitating the other encroaching  
13 species in this region. The proposal that *S. erosa* may be facilitating the growth of *S.*  
14 *burchellii* and *D. lycioides* is further strengthened by examining the sizes of these plants in  
15 the grazing plots. *S. erosa* was the tallest and had the broadest canopy diameter in the  
16 continuous-, summer- and winter-grazing plots. The large canopy diameters of *S. erosa* may  
17 be providing the seedlings of other woody species with protection against harsh temperatures,  
18 higher available soil moisture and nutrients (Tielbörger and Kadmon 2000, Chen et al. 2011,  
19 Schleicher et al. 2011). The high *S. erosa* mingling index in continuous- and summer-grazing  
20 plots supports the role of *S. erosa* as a facilitator in the growth of the other woody species.  
21 Although *S. erosa* appears to be highly competitive against grass, it had a low density in  
22 continuous- and summer-grazing plots, indicating that it is a weak competitor against *S.*  
23 *burchellii* and *D. lycioides*.

24         The larger canopy diameters of the three species in the winter grazing sub-plots  
25 relative to the continuous and summer grazing sub-plots could be due to the low shrub

density in the winter grazing plots. Plants in a high-density stand tend to vigorously compete for light and will invest more in their height than in their crown-diameter growth (Weiner and Thomas 1992, Nishimura et al. 2010, Nagashima and Hikosaka 2011). In a low-density stand, plants have the luxury of more space and less competition for sunlight and will invest in crown-diameter growth to maximise sunlight absorption (Weiner and Thomas 1992, Nishimura et al. 2010, Nagashima and Hikosaka 2011). Therefore, we expect plants in low-density stands (winter grazing sub-plots) to have a larger crown diameter compared to high-density stands (continuous and summer grazing plots) (Table 2).

The species in continuous- and summer-grazing sub-plots had a higher degree of mingling, indicating that the species were randomly distributed. However, *S. burchellii* and *D. lycioides* in the winter-grazing plots show some degree of aggregation around *S. erosa*. The aggregation of *S. burchellii* and *D. lycioides* around *S. erosa* in the winter-grazing sub-plots may indicate that the two species may maybe benefiting from higher soil-moisture content and nutrients, protection against harsh temperatures and reduced soil compaction and erosion (Tielbörger and Kadmon 2000, Flores and Jurado 2003, Schleicher et al. 2011)

*S. erosa* and *S. burchellii* showed random height and crown diameter distribution in all the grazing sub-plots. Shrubs of bigger crown diameters than itself in the continuous- and summer-grazing sub-plots generally surrounded *D. lycioides*. However, in the winter-grazing sub-plots, the Crown Diameter Differentiation (CDD) of *D. lycioides* was closer to 0.5 and the diameter was much bigger than in the continuous- and summer-grazing sub-plots (Table 2). The lower CDD of *D. lycioides* in the continuous- and summer-grazing sub-plots may indicate that *D. lycioides* is less competitive against the *Searsia* species and invests more into its height growth at the expense of its crown development to remain competitive against the other species.

1           In this study, the scale-dependence of point-pattern analysis showed that there was  
2   generally aggregation among the shrubs. These findings diverge from our prediction and  
3   those of several other studies that concluded that competition was predominant in arid and  
4   semi-arid environments (Skarpe 1991, Maestre et al. 2005). However, these findings are in  
5   agreement with those of Couteron and Kokou (1997), Tielbörger and Kadmon (2000) and  
6   Chen et al. (2011) who concluded that plants in semi-arid and desert communities tend to  
7   aggregate when small. Plants that exhibit an aggregated distribution may be facilitating the  
8   growth of one another (Schleicher et al. 2011). Plants that grow at high density may create an  
9   ‘island of fertility’, enhancing the growth of surrounding plant species, as shown in other arid  
10   environments (Ravi et al. 2010). Another reason that could lead to such aggregation is spatial  
11   heterogeneity induced by edaphic gaps (Chen et al. 2011). The gaps are formed by the  
12   removal of grass species during grazing by livestock. The period that these gaps are formed is  
13   of particular importance. When the gaps are formed in the wet season, shrub seedlings will  
14   obtain enough moisture for their establishment (Cipriotti and Aguiar 2012), and the rate of  
15   woody encroachment will be high. The high shrub density in the summer- and continuous-  
16   grazing plots in this study supports this.

17           The observed aggregation in the grazing plots could also be due to the positive effects  
18   of one plant species on the establishment of seedlings of other species (nurse-plant syndrome)  
19   which has been reported in many semi-arid and arid regions (Callaway and Walker 1997,  
20   Bruno et al. 2003). A shrub with a large canopy diameter reduces solar radiation and  
21   moderates extreme temperatures by canopy shade. The nurse syndrome was quite apparent in  
22   winter-grazing plots where *S. erosa* was the dominant species in terms of both density and  
23   crown size. The bivariate analysis showed that *S. burchellii* and *D. lycioides* were aggregated  
24   around *S. erosa*, which may indicate that *S. erosa* could be acting as a nurse plant.



## Conclusions and recommendations

Consistent with our prediction, grazing in the wet season increases the density of encroaching species. This finding may imply that grazing stock density in the wet-season plots is above the threshold limit and should be reduced. Contrary to our prediction, we generally found that there was aggregation among shrubs in the grazing plots and that *S. erosa* could be acting as a nurse plant for other shrubs.

We recognise that these findings are from an unreplicated grazing trial. We suggest that a greenhouse experiment be set up to validate these findings. We propose two greenhouse experiments. One of the experiments should test whether grazing at different intensities in the wet season causes shrub encroachment. The other experiment would test whether *S. erosa* has a nurse effect on *S. burchellii* and *D. lycopodioides* and whether this effect is due to shading, hydraulic lift and/or differential accumulation of nutrients or some combination of these factors. We also recommend a field selective-removal experiment wherein neighbors are removed to determine if the performance of the remaining target species is affected.

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**Table 1:** Species density of encroaching shrub species under different grazing regimes indicating that continuous- and summer-grazing plots have higher shrub densities than winter-grazing and continuous-rest plots. The grazing plots were open grassland at the time of establishment in 1943.

Grazing regime	Overall	<i>S. erosa</i>	<i>S. burchellii</i>	<i>D. lycioides</i>
	individuals/ha	individuals/ha	individuals/ha	individuals/ha
Continuous grazing plot 1	1624	248	768	592
Continuous grazing plot 1	1792	336	1056	400
Summer grazing plot 1	1639	165	924	550
Summer grazing plot 2	1519	250	612	562
Winter grazing plot 1	136	92	28	24
Winter grazing plot 2	176	116	40	20
Continuous rest	60	44	7	9



**Table 2:** The height, crown diameter (CD), height differentiation (HD), crown-diameter differentiation (CDD), species mingling index (MI), standardised mingling index (M) (see *Methods*) and the mean inter-shrub distance to four nearest neighbours (NND) of encroaching species in the grazing plots. EQ refers the equation number for the index.

Plot	Species	Height (m)	CD (m)	HD; EQ3	CDD; EQ3	MI; EQ1	M; EQ2	NND (m)
		Mean $\pm$ S.E	Mean $\pm$ S.E	Mean $\pm$ S.E	Mean $\pm$ S.E	Mean $\pm$ S.E	Mean $\pm$ S.E	Mean $\pm$ S.E
<b>Continuous grazing</b>	<i>S. erosa</i>	2.02 $\pm$ 0.15	2.13 $\pm$ 0.21	0.49 $\pm$ 0.01	0.48 $\pm$ 0.04	0.89 $\pm$ 0.15	0.96 $\pm$ 0.03	1.09 $\pm$ 0.18
	<i>S. burchellii</i>	1.61 $\pm$ 0.07	1.41 $\pm$ 0.09	0.46 $\pm$ 0.04	0.52 $\pm$ 0.04	0.41 $\pm$ 0.09	0.97 $\pm$ 0.01	1.25 $\pm$ 0.08
	<i>D. lycioides</i>	1.62 $\pm$ 0.09	0.99 $\pm$ 0.12	0.48 $\pm$ 0.07	0.36 $\pm$ 0.09	0.64 $\pm$ 0.17	0.87 $\pm$ 0.13	1.01 $\pm$ 0.11
<b>Summer grazing</b>	<i>S. erosa</i>	1.98 $\pm$ 0.87	2.36 $\pm$ 0.13	0.49 $\pm$ 0.06	0.54 $\pm$ 0.03	0.81 $\pm$ 0.12	0.88 $\pm$ 0.09	1.46 $\pm$ 0.12
	<i>S. burchellii</i>	1.74 $\pm$ 0.16	1.34 $\pm$ 0.08	0.46 $\pm$ 0.04	0.54 $\pm$ 0.01	0.5 $\pm$ 0.06	0.81 $\pm$ 0.04	1.42 $\pm$ 0.08
	<i>D. lycioides</i>	1.4 $\pm$ 0.54	0.99 $\pm$ 0.08	0.48 $\pm$ 0.07	0.38 $\pm$ 0.04	0.56 $\pm$ 0.12	0.97 $\pm$ 0.11	1.44 $\pm$ 0.07
<b>Winter grazing</b>	<i>S. erosa</i>	2.06 $\pm$ 0.09	3.69 $\pm$ 0.14	0.59 $\pm$ 0.06	0.62 $\pm$ 0.06	0.25 $\pm$ 0.01	0.80 $\pm$ 0.16	4.28 $\pm$ 0.13
	<i>S. burchellii</i>	1.4 $\pm$ 0.54	1.63 $\pm$ 0.23	0.5 $\pm$ 0.09	0.44 $\pm$ 0.06	0.67 $\pm$ 0.12	0.33 $\pm$ 0.02	3.34 $\pm$ 0.42
	<i>D. lycioides</i>	1.99 $\pm$ 0.2	2.39 $\pm$ 0.11	0.45 $\pm$ 0.06	0.43 $\pm$ 0.02	0.74 $\pm$ 0.09	0.24 $\pm$ 0.14	3.51 $\pm$ 0.28
<b>Continuous rest</b>	<i>Erioccephalus ericoides</i>	0.58 $\pm$ 0.16	0.62 $\pm$ 0.15		-	1	1	1.32 $\pm$ 0.1

**Table 3:** Summary of univariate analysis of three shrub species under different grazing regimes showing that the encroaching species are generally aggregated. Ag - aggregation pattern, Ra - random pattern. Numbers in parentheses are radii (in metres) at which the observed pattern is significantly different from the expected under the selected null hypotheses. L(r) is the Ripley univariate function while the O(r) is the univariate O-ring function. The univariate analysis was performed for plots that had at least 30 individuals within the plot.

Plot	Species	L (t)	O (r)
Continuous grazing 1	<i>Searsia burchellii</i>	Ag (1-12)	Ag (0-2.5) Ag (4-7)
	<i>Diospyros lycioides</i>	Ag (1-12)	Ag (0-8)
Continuous grazing 2	<i>Searsia burchellii</i>	Ag (0-2)	Ag (0-2)
	<i>Searsia erosa</i>	Ag (0-1)	Ra
	<i>Diospyros lycioides</i>	Ra	Ra
Continuous rest	<i>Eriocephalus ericoides</i>	Ag	Ag (1-10)
Summer grazing 1	<i>Searsia erosa</i>	Ra	Ra
	<i>Diospyros lycioides</i>	Ag (2-4)	Ag (1-2)
Summer grazing 2	<i>Searsia burchellii</i>	Ag (3-5)	Ag (0-5), Re (14-16)
	<i>Searsia erosa</i>	Ag (4-5), Ag (14-16)	Ra
	<i>Diospyros lycioides</i>	Ag (1-13)	Ra
Winter 1	<i>Searsia erosa</i>	Ag (6-7)	Ag (0-8)
Winter 2	<i>Searsia erosa</i>	Ra	Ag (6-7)

**Table 4:** Summary of bivariate analysis results of three shrub species under different grazing regimes showing that the encroaching species are generally aggregated. Ag - aggregation pattern, Ra - random pattern and Re - repulsion. Numbers in parentheses are radii (in metres) at which the observed pattern is significantly different from the expected under the selected null hypotheses.  $L_{12}(r)$  is the Ripley bivariate function while the  $O_{12}(r)$  is the univariate O-ring bivariate function.

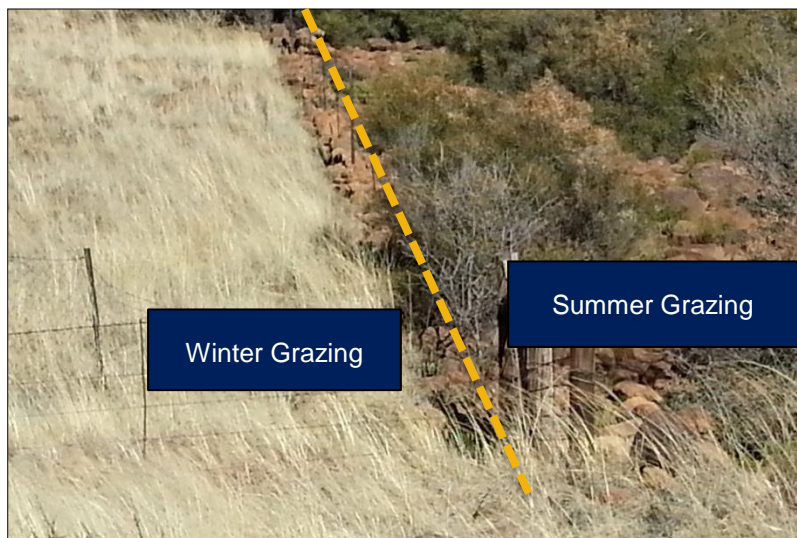
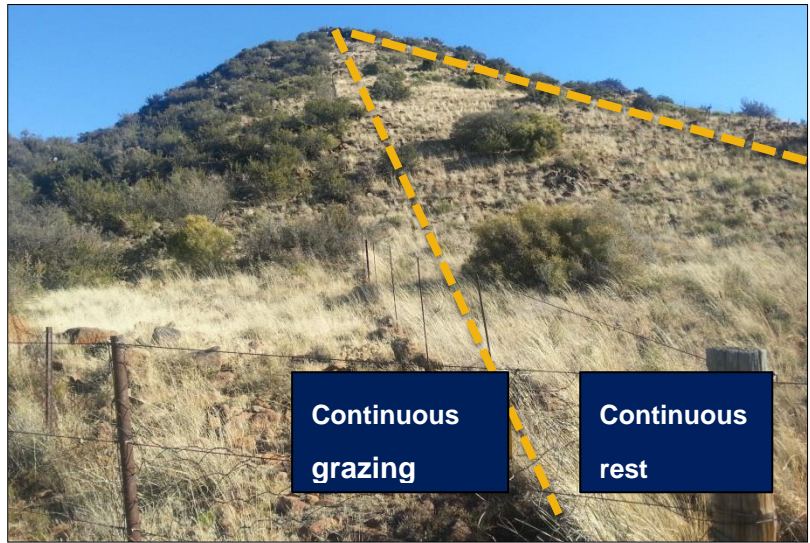
Plot	Pattern	$L_{12}(r)$	$O_{12}(r)$
Continuous grazing sub-plot 1	<i>Searsia burchellii</i> vs <i>Searsia erosa</i> and <i>Diospyros lycioides</i>	Agg (0-12)	Agg (0-7)
Continuous grazing sub-plot 2	<i>Searsia burchellii</i> vs <i>Searsia erosa</i> and <i>Diospyros lycioides</i>	Ra	Ra
Continuous grazing sub-plot 1	<i>Diospyros lycioides</i> vs <i>Searsia erosa</i> and <i>Searsia burchellii</i>	Agg (0-12)	Agg (0-7)
Continuous grazing sub-plot 2	<i>Diospyros lycioides</i> vs <i>Searsia erosa</i> and <i>Searsia burchellii</i>	Ra	Ra
Continuous grazing sub-plot 2	<i>Searsia erosa</i> vs <i>Searsia burchellii</i> and <i>Diospyros lycioides</i>	Ra	Ra
Summer grazing sub-plot 1	<i>Searsia erosa</i> vs <i>Searsia burchellii</i> and <i>Diospyros lycioides</i>	Agg (2-3)	Ra
Summer grazing sub-plot 2	<i>Searsia erosa</i> vs <i>Searsia burchellii</i> and <i>Diospyros lycioides</i>	Agg (1-8)	Agg (2-3)
Summer grazing sub-plot 1	<i>Searsia burchellii</i> vs <i>Searsia erosa</i> and <i>Diospyros lycioides</i>	Agg (2-7)	Ra
Summer grazing sub-plot 2	<i>Searsia burchellii</i> vs <i>Searsia erosa</i> and <i>Diospyros lycioides</i>	Agg (6-7)	Ra
Summer grazing sub-plot 1	<i>Diospyros lycioides</i> vs <i>Searsia erosa</i> and <i>Searsia burchellii</i>	Agg(1-7)	Agg (0-6)
Summer grazing sub-plot 2	<i>Diospyros lycioides</i> vs <i>Searsia erosa</i> and <i>Searsia burchellii</i>	Agg (0-10)	Ra
Winter grazing sub-plot 1	<i>Searsia erosa</i> vs <i>Searsia burchellii</i> and <i>Diospyros lycioides</i>	Agg (2-8)	Agg(2-3)
Winter grazing sub-plot 2	<i>Searsia erosa</i> vs <i>Searsia burchellii</i> and <i>Diospyros lycioides</i>	Re (7-25)	Ra

**Table 5:** A summary of results from the correlations between nearest neighbour distances and sum of canopy areas. \* indicates significance and *n* is the number of individuals of the respective species in the plot. N/A indicates that the values were not determined because of low sample size. There was only one sub-plot in the continuous-rest plot (see *Methods*).

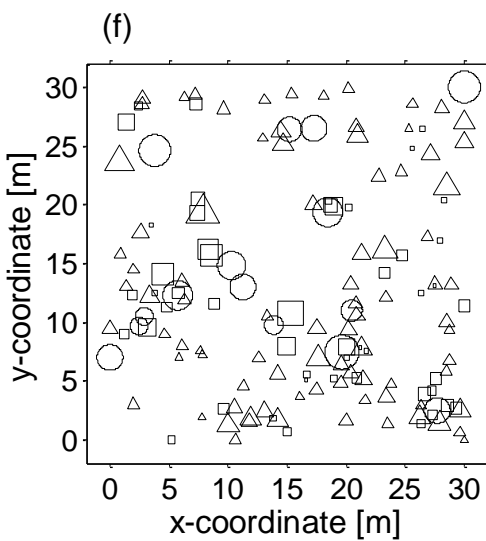
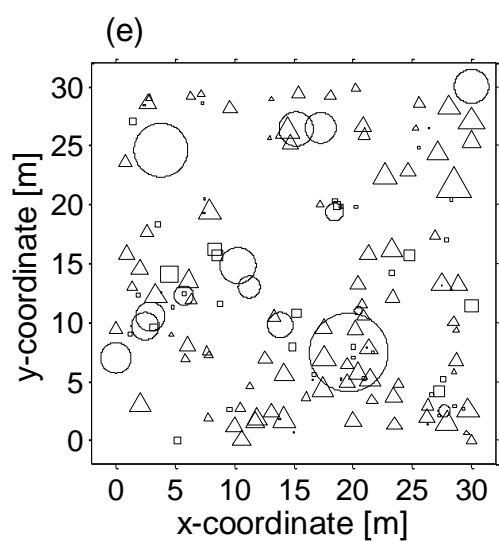
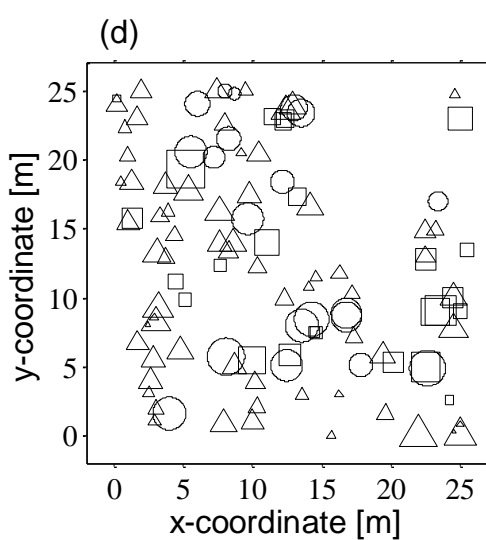
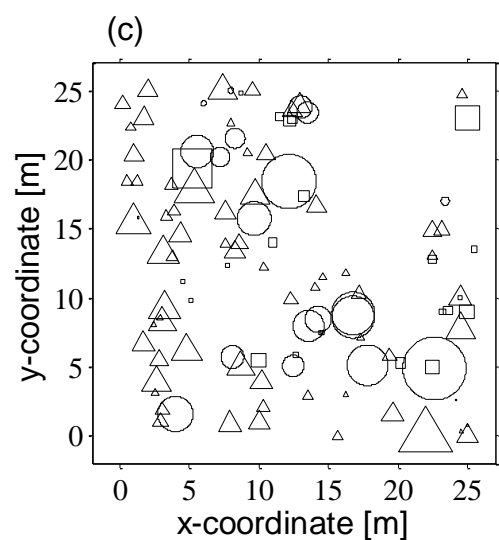
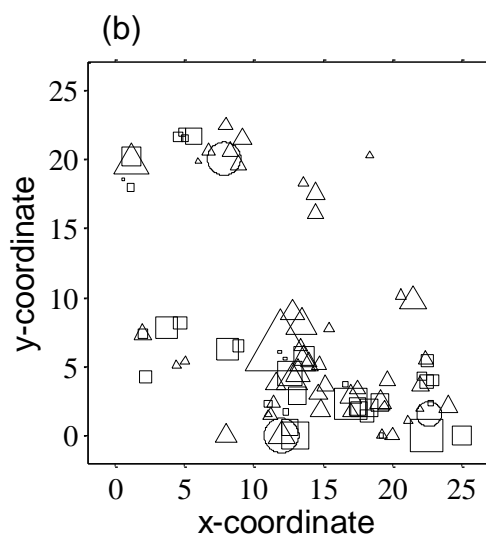
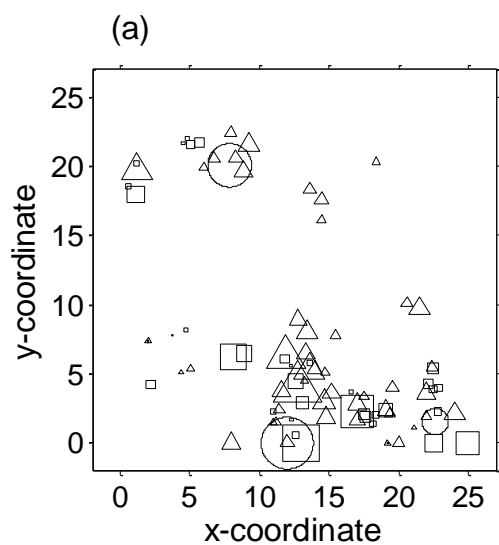
Grazing regime	Sub-plot	Species	r <sup>2</sup>	P	N
Continuous grazing	1	<i>S. erosa</i>	N/A	N/A	3
		<i>S. burchellii</i>	0.0092	0.001*	37
		<i>D. lycioides</i>	0.0147	0.001*	76
	2	<i>S. erosa</i>	0.0094	0.185	20
		<i>S. burchellii</i>	0.0017	0.004*	66
		<i>D. lycioides</i>	0.0061	0.05*	25
Summer grazing	1	<i>S. erosa</i>	0.0525	0.001*	16
		<i>S. burchellii</i>	0.0017	0.001*	84
		<i>D. lycioides</i>	0.0015	0.062	50
	2	<i>S. erosa</i>	0.0028	0.038*	40
		<i>S. burchellii</i>	0.0082	0.0001*	49
		<i>D. lycioides</i>	0.0042	0.0001*	90
Winter grazing	1	<i>S. erosa</i>	0.0016	0.02*	23
		<i>S. burchellii</i>	0.67	0.0001*	7
		<i>D. lycioides</i>	0.134	0.26	6
	2	<i>S. erosa</i>	0.064	0.0001*	28
		<i>S. burchellii</i>	0.465	0.15	10
		<i>D. lycioides</i>	0.067	0.37	6
Continuous rest		<i>E. ericoides</i>	0.143	0.0001*	78

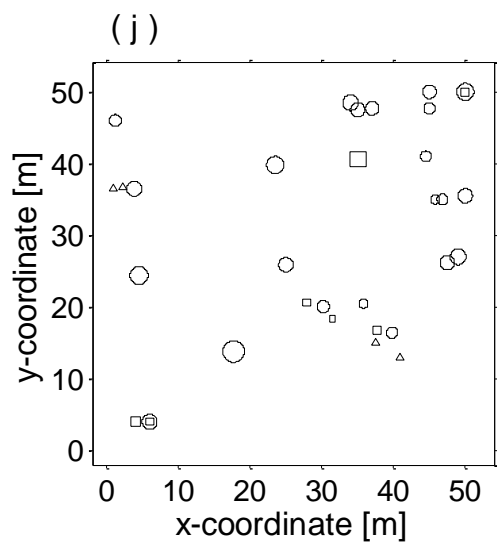
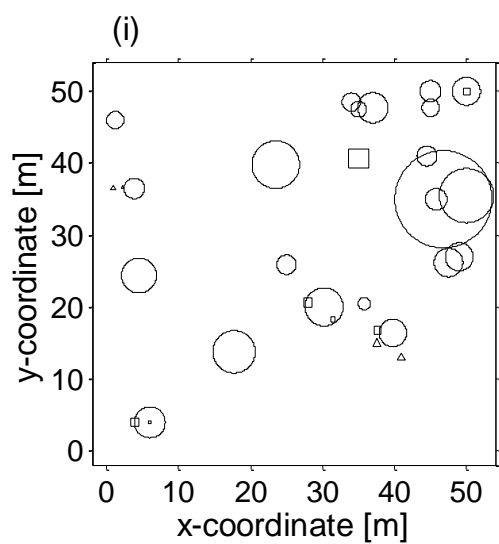
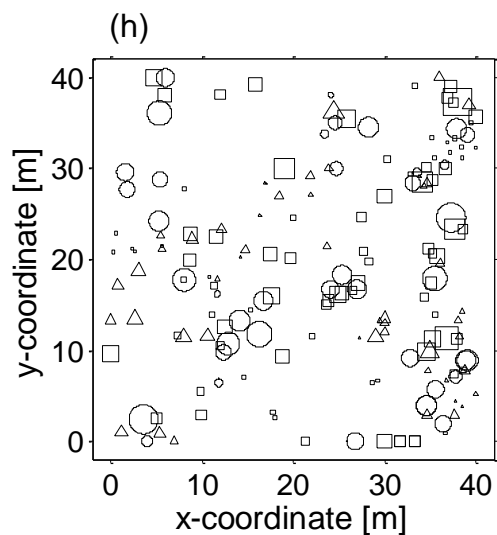
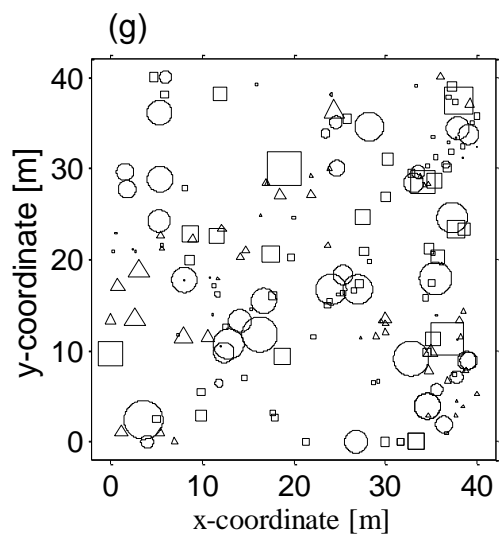


Figure 1: Aerial view of the grazing plots: (1) continuous grazing, ( 2) spring grazing, (3) summer grazing, (4) winter grazing, (5) autumn grazing, and (6) continuous-rest plots as of 30 July 2013. The study was done in the continuous, summer and winter grazing and continuous rest plots. ■ shows positions of sub-plots in each grazing plot. The sub-plots were located at mid-slope in all the grazing plots except in the continuous-grazing plot where the sub-plot was at the foot of the plot because of the limited size of this plot. The soils across the different plots were comparable.

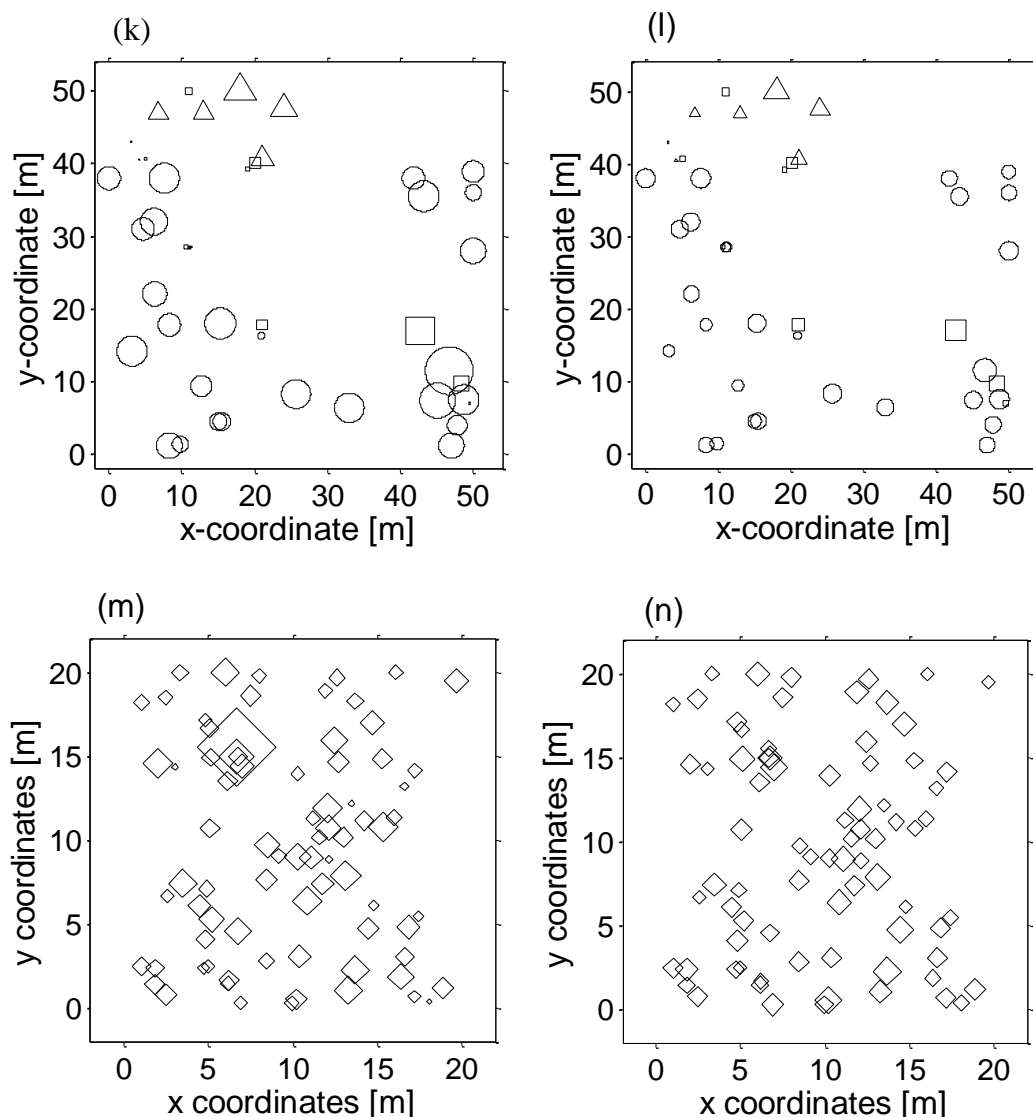


**Figure 2:** Grazing during the wet season (continuous and summer grazing plots) increases woody-plant density. The grazing density in these trial plots was two sheep per hectare.







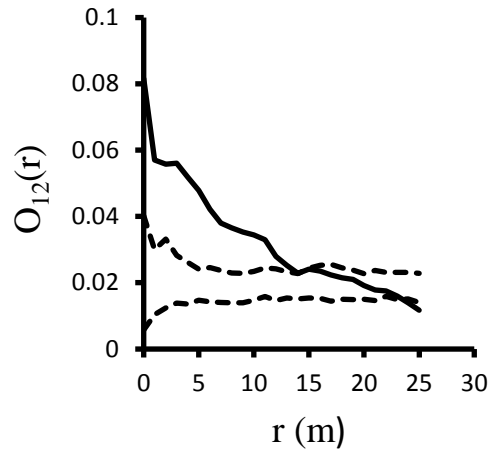
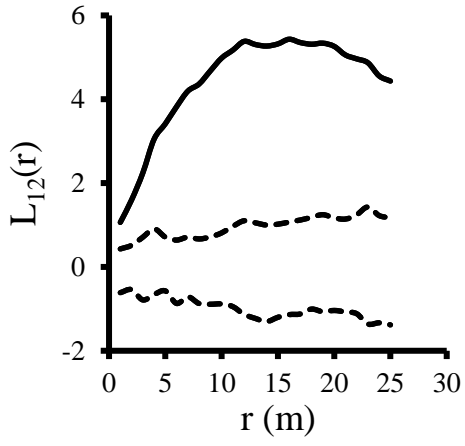


**Figure 3:** Maps of: continuous grazing sub-plot 1 with horizontal and vertical differentiation (a) and (b), respectively; continuous grazing sub-plot 2 with horizontal and vertical differentiation (c) and (d), respectively; summer grazing sub-plot 1 horizontal and vertical differentiation (e) and (f). (g) and (h) show summer grazing sub-plot 2 horizontal and vertical differentiation, respectively. In winter-grazing sub-plot 1, horizontal and vertical differentiation are shown in (i) and (j) while in winter grazing sub-plot 2 horizontal and vertical differentiation are indicated in (k) and (l), respectively. (m) and (n) represent horizontal and vertical differentiation in the continuous-rest plot, respectively. Symbols represent shrubs: *S. erosa* (○), *S. burchellii* (△), *Diospyros lycioides* (□) and *Eriocephalus*

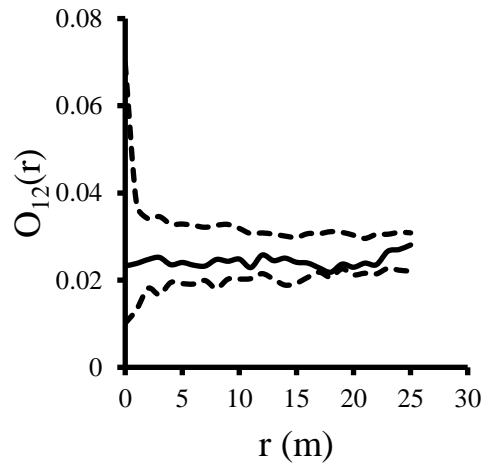
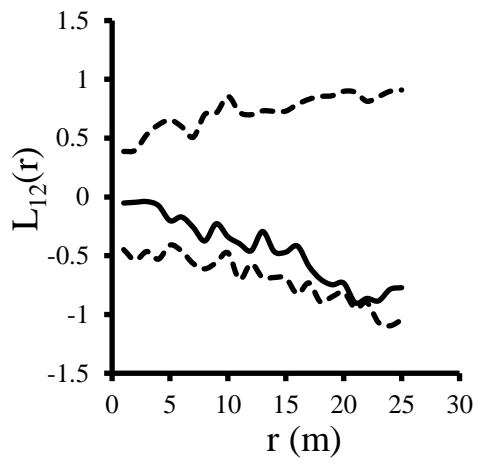
- 1 *ericoides* ( $\diamond$ ). The respective symbols are proportional to mean crown diameter and height.
- 2 The smallest diameter was 0.075 m while the largest was 13.5 m. The shortest shrub was
- 3 0.15 m while the tallest was 5.9 m.
- 4
- 5

# 1 Appendix 1

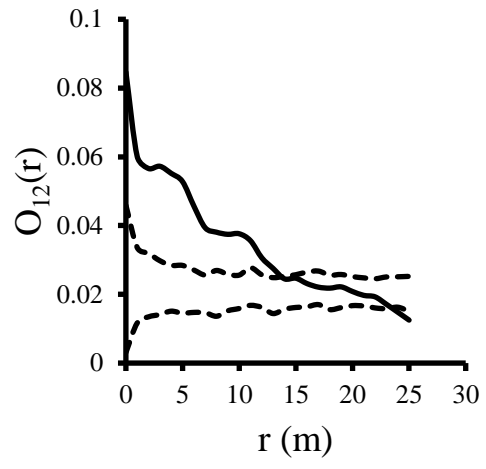
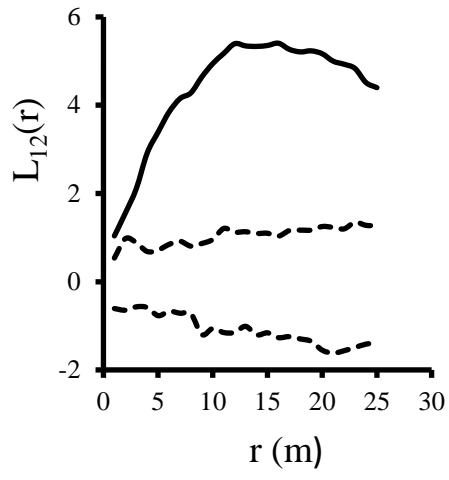
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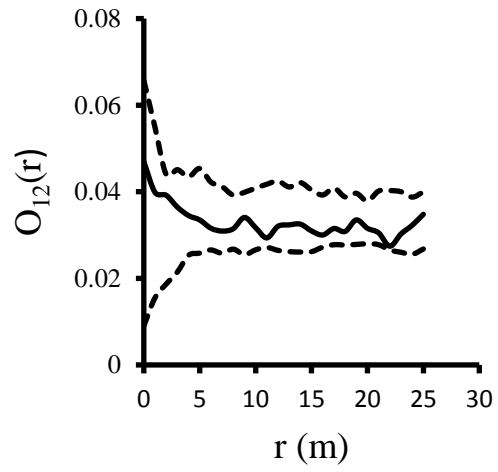
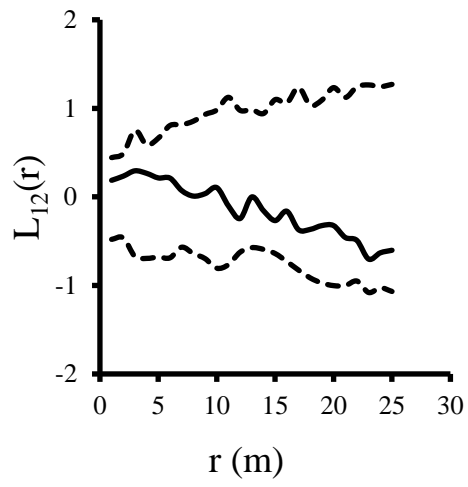
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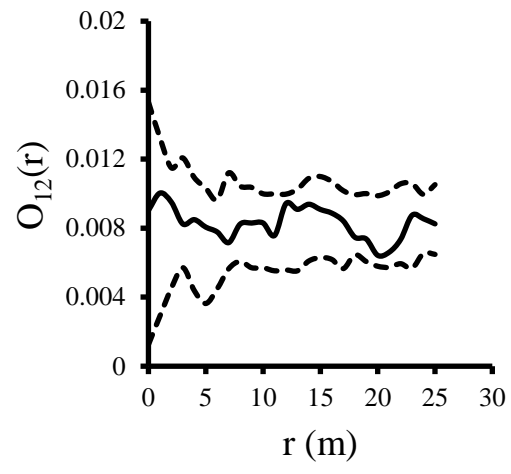
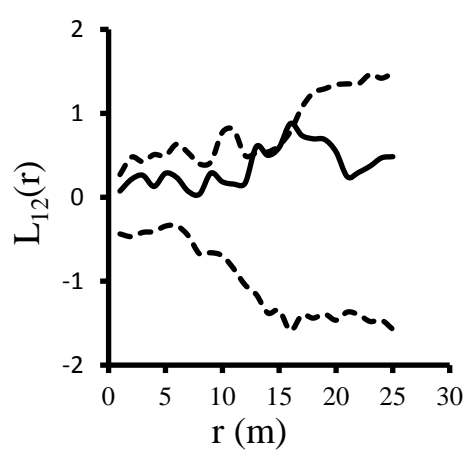
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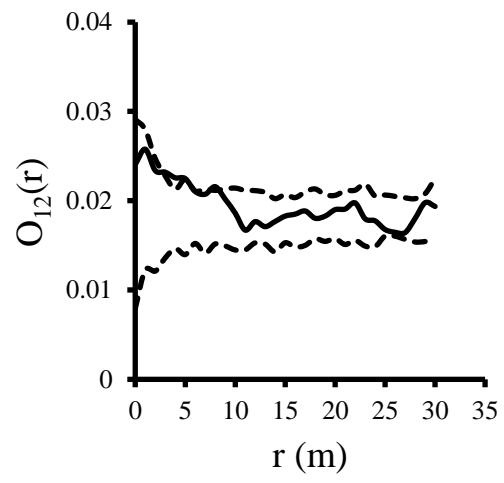
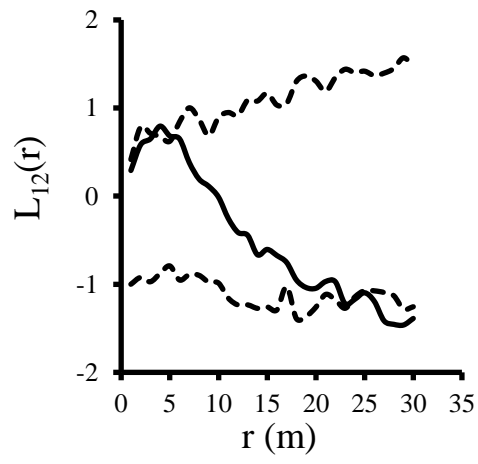
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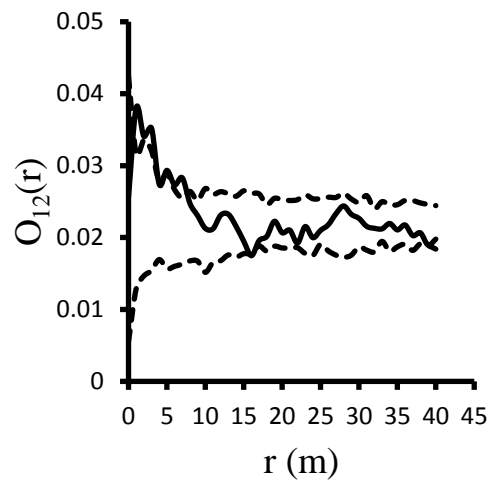
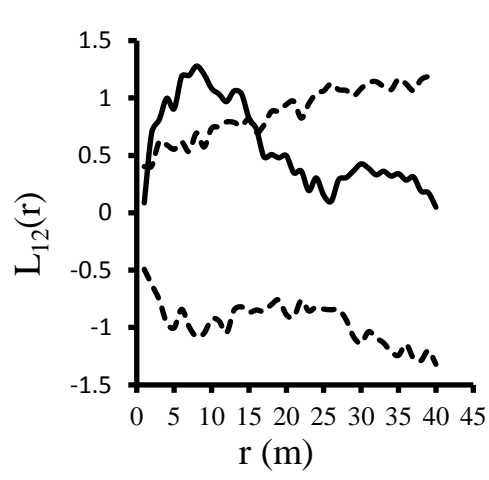
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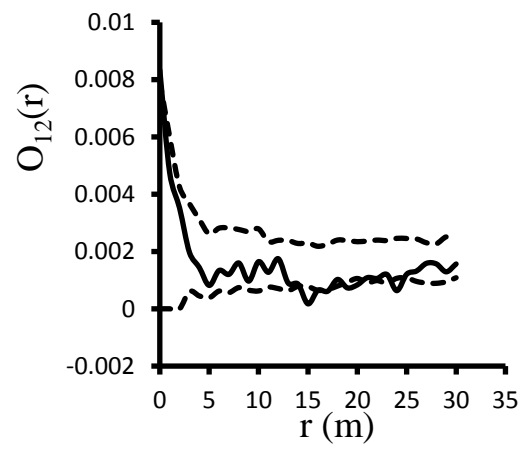
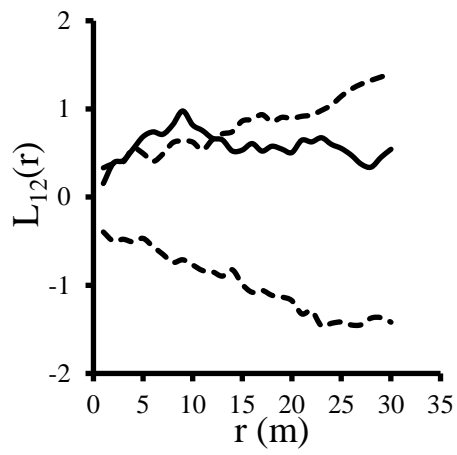
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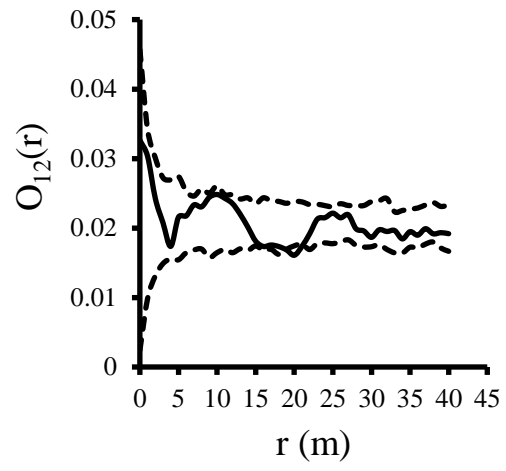
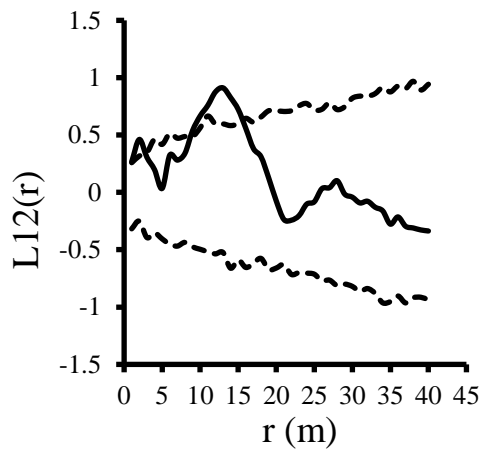
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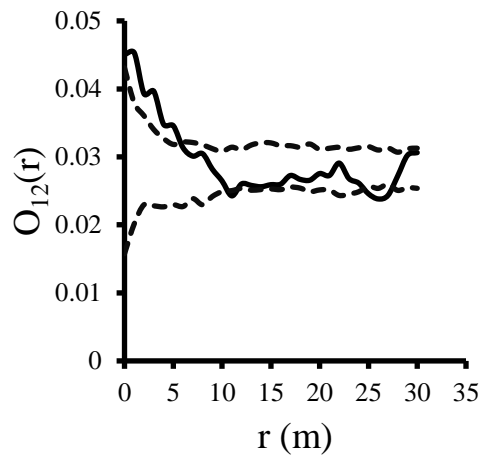
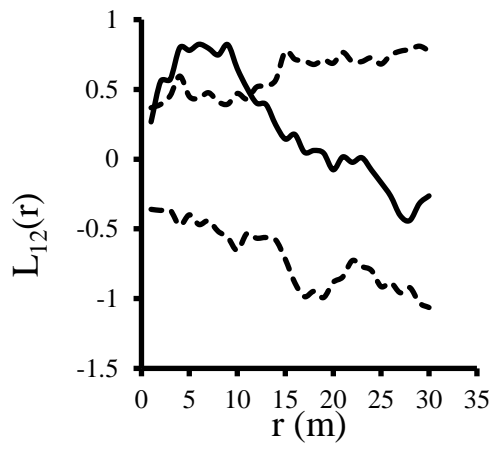
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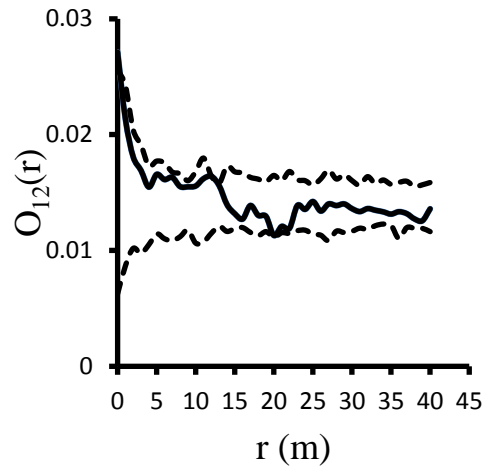
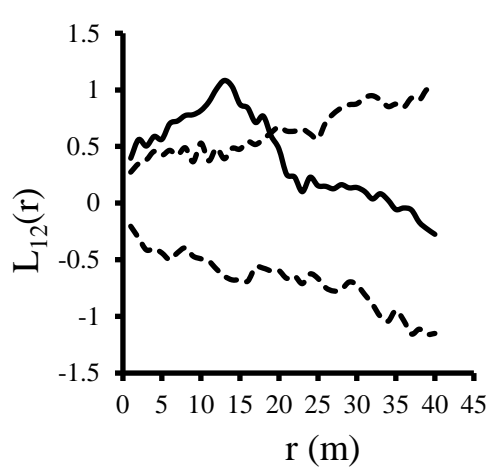
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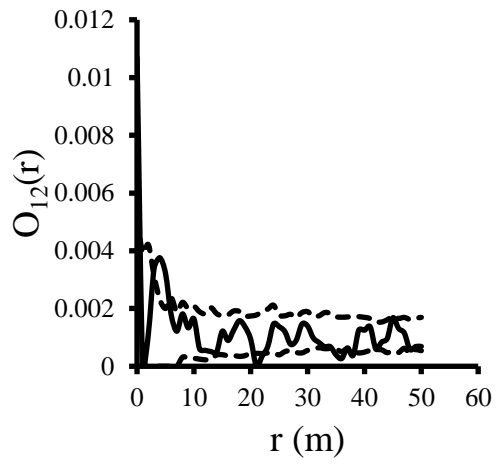
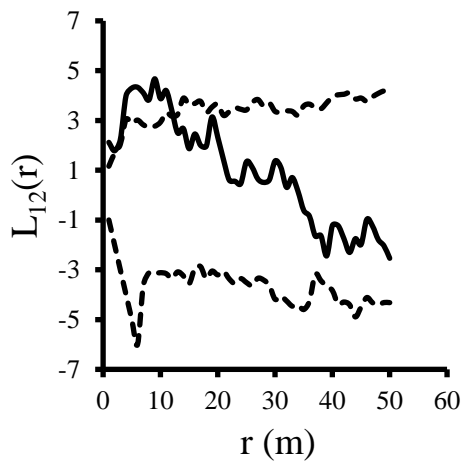
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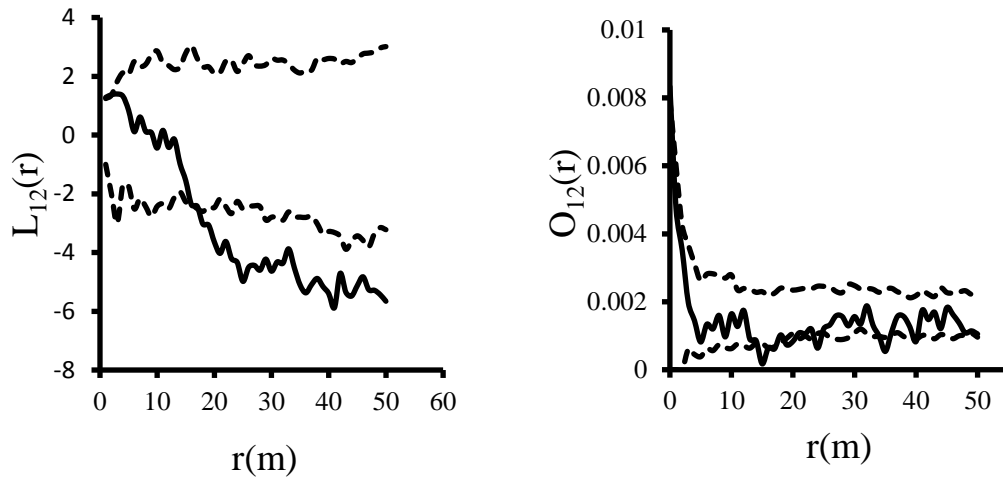


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**Appendix 1:** The bivariate  $L_{12}(r)$  function (square root transformation of the bivariate Ripley  $K_{12}(r)$  function) and the bivariate O-ring statistics  $O_{12}(r)$  showing the analysis of the interaction between *S. erosa*, *S. burchellii* and *D. lycioides* in different grazing plots.  $r(m)$  is the distance scale in metres. Observed patterns (solid line) that fall above, below and within 95 % Monte Carlo envelopes (---- dashed line) indicate aggregation, regular or random pattern, respectively. (a) Continuous-grazing sub-plot 1: *S. burchellii* vs. *D. lycioides* and *S. erosa*, (b) Continuous-grazing sub-plot 2: *S. burchellii* vs. *D. lycioides* and *S. erosa*, (c) Continuous-grazing sub-plot 1: *D. lycioides* vs. *S. erosa* and *S. burchellii*, (d) Continuous-grazing sub-plot 2: *D. lycioides* vs. *S. erosa* and *S. burchellii*, (e) Continuous-grazing sub-plot 2: *S. erosa* vs. *S. burchellii* and *D. lycioides*, (f) Summer-grazing sub-plot 1: *S. erosa* vs. *S. burchellii* and *D. lycioides*, (g) Summer-grazing sub-plot 2: *S. erosa* vs. *S. burchellii* and *D. lycioides*, (h) Summer-grazing sub-plot 1: *S. burchellii* vs. *S. erosa* and *D. lycioides*, (i) Summer-grazing sub-plot 2: *S. burchellii* vs. *S. erosa* and *D. lycioides*, (j) Summer-grazing sub-plot 1: *D. lycioides* vs. *S. erosa* and *S. burchellii*, (k) Summer-grazing sub-plot 2: *D. lycioides* vs. *S. erosa* and *S. burchellii*, (l) Winter-grazing sub-plot 1: *S. erosa* vs. *S. burchellii*

1 and *D. lycioides*, (m) Winter-grazing sub-plot 2: *S. erosa* vs. *S. burchellii* and *D. lycioides*.

2 *Note that the axes of these graphs are not the same.*

3

**CHAPTER 3: WOODY PLANT ENCROACHMENT ENHANCES CARBON  
SEQUESTRATION IN SEMI-ARID REGIONS AND DECREASES  
SEQUESTRATION IN HUMID REGIONS OF SOUTH AFRICA**

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1 Grasslands and savannas are experiencing intensive land-cover change due to shrub  
2 encroachment. In South Africa, encroachment affects more than 10 million ha, which is  
3 about 8 % of the land area of the country. This change in land cover is thought to alter  
4 soil carbon (C) and nitrogen (N) storage in these ecosystems. Some studies have  
5 reported a negative correlation between soil C and N and precipitation. There are also  
6 reports that indicate that there is no relationship with precipitation, but that there are  
7 strong relationships with soil variables such as bulk density and clay content. We  
8 quantified the changes in C and N pools and  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values to a depth of 1 m in  
9 pairs of encroached and adjacent open grassland sites along a precipitation gradient  
10 from 300 mm to 1500 mm per annum in South Africa. Our study showed a negative  
11 correlation between changes in soil organic C in the 0-100 cm soil layer and mean  
12 annual precipitation (MAP). The most humid site (1500 mm MAP) had less C in shrub-  
13 encroached sites while the drier sites (300-350 mm MAP) had more C than their paired  
14 open grasslands. This study generally showed soil organic C gains in low precipitation  
15 areas, with a threshold value between 700 mm and 900 mm. Our threshold value was  
16 higher than that found in North America, suggesting that one cannot extrapolate across  
17 continents.

18 Soils are a major reservoir of terrestrial carbon (C), storing more C (2 344 Gt C up to 3 m  
19 depth) than terrestrial biomass (560 Gt C) and atmospheric pools (750 Gt C)<sup>1, 2, 3</sup>. A small  
20 change in soil organic C (SOC) may have a great impact on atmospheric C and subsequently  
21 on earth's climate<sup>4</sup>. Land-cover change is one of the main factors that can alter SOC  
22 content<sup>5, 6</sup>. One of the major sources of land-cover change, especially in tropical and  
23 subtropical regions, is woody plant encroachment. In South Africa, this problem is  
24 particularly acute; encroachment affects about 10-20 million ha, seriously reducing the  
25 productivity for a country where >70% of its agricultural area is largely grazing lands<sup>7</sup>.

Jackson et al.<sup>8</sup> examined the effects of vegetation change at six paired grassland and invaded woody sites along a precipitation gradient in the south-western USA, testing relationships between biomass C gains and SOC losses for common native woody invaders, such as *Prosopis* (mesquite), *Larrea* (creosote) and *Juniperus* (juniper) species. In that study, they found that there was a greater increase in SOC stored in the encroached sites than in the open grasslands in drier locations (i.e., below ~400 mm mean annual precipitation (MAP)). However, Jackson et al.<sup>8</sup> also observed lower values of SOC in encroached sites than in open grasslands in the more humid locations. Other data show that generally organic C and total N pools in soils beneath shrub canopies increase linearly with time since shrub establishment<sup>9</sup>. A subsequent meta-analysis of SOC data on woody plant encroachment found that the change in soil organic C in response to shrub encroachment was highly variable and unrelated to MAP<sup>10</sup>.

Soil bulk density and clay content mediate the magnitude and direction of change in SOC with woody encroachment. Soil carbon accumulation when woody plants encroach decreases as soil bulk density increases<sup>10</sup>. SOC accumulates linearly with woody plant encroachment with increasing clay content<sup>11, 12</sup>. Barger *et al.*<sup>10</sup> reported that SOC accumulated linearly with increasing clay content ( $r^2 = 0.76$ ), presumably reflecting greater occlusion and protection of organic matter afforded by clay micelles<sup>11, 12</sup>. They also found that soil C accumulation decreased as soil bulk density increased, with C gains ( $r^2 = 0.52$ ) being confined to soil densities less than  $1.6 \text{ g cm}^{-3}$ .

Woody plant encroachment in tropical grasslands usually alters the ecosystem from a  $C_4$  to a  $C_3$  dominated photosynthetic pathway<sup>13, 14</sup>. The change in the dominant photosynthetic pathway offers a unique opportunity to use the natural abundance of  $^{13}\text{C}$  to evaluate changes in C cycles due to woody plant encroachment of grasslands. Variations in

$^{13}\text{C}/^{12}\text{C}$  ratios of plants utilising the  $\text{C}_3$  and  $\text{C}_4$  photosynthetic pathways provides a natural tracer when a  $\text{C}_4$  community type ( $\delta^{13}\text{C} \approx -14 \text{ ‰}$ ) is replaced by a  $\text{C}_3$  community ( $\delta^{13}\text{C} \approx -27 \text{ ‰}$ ) or *vice versa*<sup>13, 14</sup>, allowing the quantification of the C derived from the pre-shrub encroachment vegetation and C derived from the current vegetation<sup>8, 15, 16</sup>.

SOC in encroached grasslands can also be enhanced by an increase in available soil nitrogen. Change in nitrogen availability in ecosystems is likely to drive net primary productivity and thus C sequestration<sup>17</sup>. The frequent increase of  $\text{N}_2$ -fixing-woody species in grasslands<sup>18, 19</sup> has strong potential for altering the N-cycle, primary production, and other key ecosystem processes<sup>19, 20</sup>. Plant species are likely to influence ecosystem N cycling via alterations in N-use efficiency and by changing N inputs and losses<sup>21</sup>. Changes to the soil total N pool via the shift in the balance of N inputs versus losses should be apparent in the natural abundance of  $^{15}\text{N}$  in the plant-soil system<sup>19</sup>.

In this study, we quantified and compared C and N pools (measured as concentrations and mass stocks) between adjacent woody- encroached and open-grassland plots along a precipitation gradient (300 mm to 1500 mm per annum) in South Africa. The major encroaching species in our sites (except in Middelburg) were potentially nitrogen-fixing shrubs (*Acacia karroo*, *A. mellifera*, *A. tortilis* and *A. erioloba*). We focused on responses in C sequestration, and in particular its relationship with MAP, as a key continental scale driver of ecosystem function<sup>22</sup>. We recorded SOC, N,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  at depth intervals of 0-10, 10-30, 30-60 and 60-100 cm and determined the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  isotopic ratio in plant leaves and soil litter. We also determined the contribution of  $\text{C}_4$  plants to SOC in the encroached grasslands. Our study sites did not include any herbaceous  $\text{C}_3$  plants. The sites in decreasing order of precipitation were KwaMbonambi (1500 mm  $\text{yr}^{-1}$ ), Stanger (900 mm  $\text{yr}^{-1}$ ), Bergville (700 mm  $\text{yr}^{-1}$ ), Bloemfontein (500 mm  $\text{yr}^{-1}$ ), Pniel (350 mm  $\text{yr}^{-1}$ ) and Middelburg

(300 mm yr<sup>-1</sup>). At each site, we had six encroached and six open grasslands. We predicted a negative correlation between the magnitude of the increase in C with the degree of encroachment and MAP<sup>8</sup>, as well as a negative correlation between C change due to woody encroachment and soil bulk density and a positive correlation between C change due to woody encroachment and silt and clay content<sup>10</sup>. We predicted similar relationships for soil N because of the tight relationship between soil C and N<sup>8</sup>. We also predicted that  $\delta^{13}\text{C}$  will get more enriched with depth because of the transition from C<sub>4</sub> to C<sub>3</sub> vegetation<sup>15, 16</sup> and also because of discrimination against <sup>13</sup>C during microbial respiration<sup>23, 24</sup>.

At the most humid site (1500 mm MAP), in the 0-10 cm layer, soil C concentration was significantly greater ( $F_{1, 10} = 83.70$ ,  $P < 0.0001$ ) in the open grasslands than in shrub-encroached grasslands (Table 1, Figure 1). Open grasslands at this humid site lost 50.5 % of soil organic carbon stocks in the uppermost soil layer. The loss in SOC stocks to 100 cm depth due to woody plant encroachment at this humid site was almost 50 %. At the 900, 700 and 500 mm MAP sites there were no differences in soil C concentrations between encroached and open grasslands in the uppermost soil layer (Figure 1). However, the 700 and 500 mm MAP sites gained carbon stocks slightly at 0-1 m depth, ranging between 9.2% and 25%. In the semi-arid sites of 350 and 300 mm MAP, soil organic C concentration in the 0-10 cm soil layer was higher ( $F_{1,10} = 5.06$ ,  $P = 0.048$  and  $F_{1,10} = 16.73$ ,  $P = 0.002$ , respectively) in the open grassland than in the paired encroached grasslands (Figure 1). The 350 and 300 mm MAP sites had higher soil C concentrations in the encroached grasslands compared to open grasslands with increasing depth. There was a general decrease of SOC with depth across our entire sites in both encroached and unencroached grasslands.

There was a significant negative correlation between change in C stocks in open versus encroached grasslands in the 0-100 cm soil layer and mean annual precipitation (MAP) ( $r = -0.91$ ,  $P = 0.012$  (Fig 2a)). In contrast, there was no significant relationship

between change in C stocks (between open and encroached grasslands) and soil bulk density ( $r = -0.22$ ,  $P = 0.68$  (Fig 2b)) and between change in C stocks and silt and clay content ( $r = 0.14$ ,  $P = 0.77$  (Fig 2c)).

Nitrogen was significantly higher in shrub-encroached grasslands than in the open grasslands in the 0-10 cm soil layer in the 900, 500, 350 and 300 mm (Supplementary material - Figure 4). The correlation between change in total N stocks of encroached versus open grasslands in the 0-100 cm soil layer and MAP tended to be positive ( $r = 0.79$ ,  $P = 0.064$ ; data not shown). There was no relationship between total N change between encroached and open grasslands and bulk density ( $r = 0.095$ ,  $P = 0.89$ ) and between total N change and silt and clay content ( $r = 0.16$ ,  $P = 0.76$ ).

In the encroached grasslands,  $\delta^{13}\text{C}$  was generally more negative in the plant material (mixture of foliage and stems) compared to the top soil. The open grassland showed the opposite trend with the plant material being more enriched in  $\delta^{13}\text{C}$  than the top soil (supplementary material, Figure 5).  $\delta^{15}\text{N}$  was generally more enriched in the top soil compared to the plant material in both the encroached and open grasslands (Figure 5).

At 0-10 cm depth, the  $\delta^{13}\text{C}$  values were significantly different between encroached and open grasslands in the 1500, 900, 500 and 350 mm MAP sites while in the 300 and 700 mm MAP sites there was no significant difference in  $\delta^{13}\text{C}$  between the two encroached and open grasslands (Figure 3). There was generally  $\delta^{13}\text{C}$  enrichment with increasing depth across the precipitation gradient. There was no significant correlation between change in  $\delta^{13}\text{C}$  between encroached and open grasslands and mean annual precipitation (MAP) ( $r = 0.17$ ,  $P = 0.35$ ). There was no significant enrichment of  $\delta^{15}\text{N}$  with depth in both the encroached and open grasslands (supplementary material - Figure 6) across all sites. Encroached grasslands



1 had a significantly higher  $\delta^{15}\text{N}$  than open grasslands in the 500 MAP site ( $F_{1,10}=8.72$ ,  $P =$   
2  $0.01$ ) in the 0-10 cm soil layer (supplementary material Figure 6).  $\delta^{15}\text{N}$  change between  
3 encroached and open grasslands was also not correlated with MAP ( $r = 0.45$ ,  $P = 0.46$ ).  
4 Although there was a positive relationship between  $\delta^{15}\text{N}$  change and silt and clay content, the  
5 relationship was marginally significant ( $r = 0.80$ ,  $P = 0.06$ ). However, we found a significant  
6 correlation between  $\delta^{15}\text{N}$  change and bulk density ( $r = -0.93$ ,  $P = 0.008$ ).

7         We acknowledge that the isotopic-mixing model used here does not take into account  
8 the fractionation of  $\delta^{13}\text{C}$  with depth due to decomposition<sup>24</sup>. Assuming that there is no  
9 isotopic fractionation during decomposition<sup>23,25</sup>, the isotopic-mixing model indicated that the  
10 percentage contribution of  $\text{C}_4$  plants (i.e. most grasses in these hot environments) to SOC in  
11 the encroached grassland decreased from 75.8 % in the 0-10 cm soil layer to 68.3 % in the  
12 60-100 cm soil layer at the 1500 mm MAP site. From the 900 to the 300 mm MAP sites, the  
13 contribution of  $\text{C}_4$  plants to SOC increased from 35.4 % in the 0-10 cm layer to over 80 % in  
14 the 60-100 cm soil layer.

15         Our study lacked a direct test of SOC change independent of other covarying factors,  
16 such as fire, grazing intensity and microbial activities. Nonetheless, our findings are in  
17 agreement with a number of studies<sup>8,26,27</sup> that found a negative correlation between change in  
18 SOC and MAP due to shrub encroachment. Our results, however, were contrary to a review  
19 by Barger et al.<sup>11</sup> who showed that C response to shrub encroachment was highly variable  
20 and unrelated to MAP. Additionally, our findings also show that shrub encroachment  
21 sequesters more C at MAP < 900 mm while the study by Jackson and colleagues<sup>8</sup> found a  
22 threshold value of approximately 400 mm MAP. The variance in the threshold MAP might be  
23 due to large differences in SOC level between their studies and our study sites, with their  
24 most arid site (230 mm MAP) having more C than our 500 mm MAP site.

1           The increase of SOC in the drier sites is in agreement with the well-described island  
2 of fertility<sup>12, 28</sup>, where shrubs and trees develop extensive root systems that extract nutrients  
3 from the surroundings, leading to local nutrient recycling and greater biomass production  
4 associated with organic matter accumulation<sup>29</sup>. The accumulation of SOC in the drier shrub-  
5 encroached grasslands can also be due to greater rates of primary productivity in the more  
6 arid sites because the encroached grasslands had greater biomass than open grasslands.  
7 Greater primary productivity increases soil organic matter input in the soil beneath deep-  
8 rooted shrubs through increased surface litter and accumulation of complex organic  
9 compounds, which are resistant to decomposition<sup>12, 22</sup>. The humid KwaMbonambi site  
10 (MAP = 1500 mm) had more C in the open grassland compared to the encroached grassland.  
11 This is consistent with the findings of Jackson et al.<sup>8</sup>. In high-precipitation areas, grasslands  
12 are highly productive and allocate a large proportion of SOC belowground with greater root  
13 turnover rates<sup>8</sup>. The differences in  $\delta^{13}\text{C}$  between the aboveground plant material and the top  
14 soil in encroached grasslands indicate that the source of the SOC is not only from the present  
15 aboveground plant material.

16           The change in  $\delta^{13}\text{C}$  with depth across the precipitation gradient could be due to kinetic  
17 discrimination against  $^{13}\text{C}$  during respiration<sup>23</sup> or it could be an indication of different sources  
18 of carbon over time<sup>30</sup>. Although the encroached grasslands are dominated by  $\text{C}_3$  plants, the  
19 bulk of the SOC still appears to come from the  $\text{C}_4$  plants according to the mixed-isotopic  
20 model we used. This also assumes that there is no significant discrimination against  $^{13}\text{C}$   
21 during respiration/decomposition<sup>23, 25</sup>. The increase in the  $\text{C}_4$  contribution to SOC with depth  
22 to over 80 %  $\text{C}_4$  values may be a great indication that these encroached sites were once open  
23 grassland. We have shown elsewhere, using aerial and fixed-point photography, that several  
24 of the sites (300 mm, 350 mm and 700 mm MAP) were open grasslands 20 to 50 years

ago<sup>31,32,33,34</sup>.

Soil N was higher in the encroached grasslands across all sites over the 0-100 cm soil depth probably because most of the encroaching woody species are leguminous N-fixing plants such as *Acacia erioloba*, *A. karroo* and *A. mellifera*. Nitrogen-fixing plants ameliorate and increase N in the soil<sup>35</sup>. This finding is consistent with results from other African savanna ecosystems where sites encroached by woody species have been compared with open grasslands<sup>32, 36, 37</sup>. Another plausible mechanism for increased N inputs in encroached grassland soils is atmospheric deposition. The greater canopy height and leaf area in encroached grasslands vs. open grasslands may be more effective in capturing and trapping atmospheric N-deposition, resulting in greater accumulation of this source in wooded areas<sup>19</sup>. The isotopic composition of N derived from atmospheric deposition is generally depleted in <sup>15</sup>N. Thus, inputs of biologically-fixed and atmosphere-deposited N may be responsible for the decrease in soil  $\delta^{15}\text{N}$  values following woody plant encroachment into grasslands in our mesic sites (500 and 700 mm MAP). Although foliage and surface litter in our sites showed some evidence of nitrogen fixation in the encroached grasslands, the  $\delta^{15}\text{N}$  values did not decrease with soil depth as would be expected if the source of the increase in soil N pools was N fixation rather than atmospheric N-deposition<sup>19, 30</sup>. However, this soil-depth effect might have been neutralized by other processes affecting <sup>15</sup>N concentration, such as N pumping from deeper soil layers and isotope fractionation during litter decomposition<sup>19, 38</sup> and denitrification<sup>39</sup>.

This study generally showed soil organic C gains in low precipitation areas, with a threshold value between 750 mm and 900 mm MAP due to shrub encroachment and that most of the SOC in the shrub-encroached grasslands came from the C<sub>4</sub> plants which previously occupied the area. The significant difference between our study and the North American study of Jackson et al<sup>8</sup>. which showed a threshold of 400 mm indicates that

1    extrapolation across continents is problematic, as has been indicated for savannas  
2    elsewhere<sup>40</sup>.

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## Figure legend

Figure 1 Soil organic carbon concentrations ( $\text{g C kg}^{-1}$  soil) vs. depth (cm) along a precipitation gradient (300-1500 mm MAP) in encroached grasslands (●) and open grasslands (○). Error bars represent standard errors. Some encroached and open grassland data points on the 350 and 300 mm sites completely overlap.

Figure 2 Relationship between changes in SOC stocks (%) at 0-100 cm depth with woody encroachment in pairs of encroached and open grasslands and (a) mean annual precipitation (MAP) in mm; (b) silt and clay content as percentage; (c) bulk density in  $\text{g cm}^{-1}$ . A positive value is achieved because there is more SOC in encroached soils than in open grassland soils and *vice versa*.

Figure 3  $\delta^{13}\text{C}$  values of soil organic carbon in encroached grassland (●) and open grasslands (○) with depth (cm) in South Africa. Error bars are standard errors of the mean. The reference standard was Pee Dee Belemnite.

## Methods

### Soil sampling of soil organic carbon and nitrogen

Soil samples were collected between October 2013 and March 2014. Each site was divided into encroached and unencroached sections, using adjacent sections to minimize differences in topography. The goal of this paired experimental design was to compare the effects of herbaceous and woody communities without initial differences in soil properties and vegetation cover. In three of our sites (Middelburg, Pniel and Bergville) there is documented evidence<sup>31, 32, 33, 34</sup>, while for the other three sites we have strong oral evidence suggesting that our sites were once open grasslands 20 – 50 years ago. Encroached sites were defined as those areas with more than 40 % shrub cover. Six 1 m-deep and 1 m wide pits were dug at random at each site in each section. Within each pit, two soil cores were sampled (drilled horizontally into the side of the pit) at four different depths (0-10, 10-30, 30-60 and 60-100 cm). One core was used to quantify bulk density (BD) and soil texture, and the other was used to determine soil organic carbon (SOC), total nitrogen (TN),  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values. Bulk density and soil texture were quantified using the core method and pipette method, respectively<sup>41, 42</sup>. The soil cores designated for SOC, TN,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  were air-dried and passed through a 2 mm sieve. To remove soil inorganic C, soil samples were treated with 1N  $\text{H}_2\text{SO}_4/5\% \text{FeSO}_4$  and double-checked for complete inorganic C removal. Soil samples were then air-dried at 40 °C and then sieved (1-mm mesh). Generally, our soil samples were free of rocks. Total SOC and N were measured using a Europar elemental analyser at BemLabs (Somerset West, Western Cape). The SOC and N stocks were calculated using the following equation:

$$S_s = x_1 x_2 x_3 \quad (1)$$

where  $S_s$  is the SOC or nitrogen stocks ( $\text{Mg C ha}^{-1}$ );  $x_1$  is the C or N concentration in the soil material (%);  $x_2$  is the soil bulk density ( $\text{Mg m}^{-3}$ );  $x_3$  is the thickness of the soil layer (cm).

For  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values, c. 40 mg of soil was weighed into tin capsules (Elemental Microanalysis Ltd., Devon, UK) and combusted in a Thermo Flash EA 1112 series elemental analyser; the gases were fed into a Delta Plus XP isotope ratio mass spectrometer (Thermo Electron Corporation, Milan, Italy). The C isotopic ratio of a sample were expressed relative to the Pee Dee Belemnite standard<sup>43</sup> and the N isotopic ratios was expressed relative to atmospheric nitrogen<sup>44</sup>.

Carbon isotope ratios are presented in  $\delta$ -notation:

$$\delta^{13}\text{C} = [(R_{\text{SAMPLE}} - R_{\text{STD}})/R_{\text{STD}}] \times 10^3 \quad (2)$$

where  $R_{\text{SAMPLE}}$  is the  $^{13}\text{C}/^{12}\text{C}$  ratio of the sample, and  $R_{\text{STD}}$  is the  $^{13}\text{C}/^{12}\text{C}$  ratio of the Pee Dee Belemnite standard<sup>43</sup>.

Nitrogen isotope ratios are also presented in  $\delta$ -notation:

$$\delta^{15}\text{N} = [(R_{\text{SAMPLE}} - R_{\text{STD}})/R_{\text{STD}}] \times 10^3 \quad (3)$$

where  $R_{\text{SAMPLE}}$  is the  $^{15}\text{N}/^{14}\text{N}$  ratio of the sample, and  $R_{\text{STD}}$  is the  $^{15}\text{N}/^{14}\text{N}$  ratio of the atmospheric N standard.

Assuming complete and unbiased mixing in the soil, we used the  $\delta^{13}\text{C}$  values of soils and the mean  $\delta^{13}\text{C}$  values for the foliage of  $\text{C}_3$  (shrub) and  $\text{C}_4$  (grass) species present at our sites to estimate the relative proportion of soil organic matter derived from  $\text{C}_4$  and  $\text{C}_3$  photosynthetic pathway sources with an isotopic-mixing model<sup>8</sup>:

$$\text{FC}_4 = (\delta^{13}\text{C}_{\text{soil}} - \delta^{13}\text{C}_{\text{C}_3}) / (\delta^{13}\text{C}_{\text{C}_4} - \delta^{13}\text{C}_{\text{C}_3}) \quad (4)$$

where  $\text{FC}_4$  is the carbon fraction derived from  $\text{C}_4$  sources,  $\delta^{13}\text{C}_{\text{soil}}$  is the measured  $\delta^{13}\text{C}$  of the soil sample,  $\delta^{13}\text{C}_{\text{C}_4}$  is the mean  $\delta^{13}\text{C}$  of  $\text{C}_4$  sources, and  $\delta^{13}\text{C}_{\text{C}_3}$  is the mean  $\delta^{13}\text{C}$  of  $\text{C}_3$  sources<sup>31</sup>. The isotopic mixing model used here does not take into account  $\delta^{13}\text{C}$  fractionation with depth due to decomposition. Several studies have shown no indication of isotopic

fractionation during decomposition<sup>23, 25</sup>, while several other studies have shown various <sup>13</sup>C fractionations due to decomposition<sup>24, 45</sup>.

The data on C and N concentration in soil, litter, root, and SOC stocks  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values attributable to the effects of shrub encroachment and soil depth were analysed using a multivariate ANOVA analysis (MANOVA) to reduce problems with Type I error because we examined several dependent variables simultaneously.

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1 **Author contribution**

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3 All authors designed, wrote and edited the manuscript. A.M. collected and analysed the data.

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1    **Competing financial interest**

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3    The authors declare no competing financial interests.

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Table 1 Soil organic C and N stocks (Mg/ha) to 1 m depth and % change in C and N after shrub encroachment.

MAP (mm)	0-10 cm			10-30 cm			30-60 cm			60-100			0-100 cm		
	Shrub	Grass	%change	Shrub	Grass	%change	Shrub	Grass	%change	Shrub	Grass	% change	Shrub	Grass	% change
<b>Soil organic carbon stocks</b>															
1500	4.9	9.9	<b>-50.5</b>	6.0	10.5	<b>-43.5</b>	5.9	9.8	<b>-40.4</b>	3.5	9.9	<b>-64.4</b>	20.3	40.2	<b>-49.6</b>
900	27.6	30.5	<b>-9.5</b>	25.3	33.6	<b>-24.7</b>	17.1	23.3	<b>-26.4</b>	21.6	13.5	<b>59.9</b>	91.6	100.9	<b>-9.2</b>
700	40.0	48.7	<b>-18.0</b>	42.1	35.2	<b>19.5</b>	55.8	38.7	<b>44.3</b>	62.1	36.5	<b>70.4</b>	200.1	159.1	<b>25.7</b>
500	16.0	12.8	<b>24.8</b>	21.2	16.0	<b>32.4</b>	19.1	24.6	<b>-22.4</b>	23.1	19.0	<b>21.7</b>	79.4	72.4	<b>9.7</b>
350	7.8	5.0	<b>55.9</b>	6.1	6.0	<b>2.2</b>	6.7	6.6	<b>1.7</b>	6.9	7.0	<b>-0.5</b>	27.5	24.5	<b>12.3</b>
300	8.2	3.9	<b>108.9</b>	6.4	6.1	<b>5.2</b>	8.2	6.0	<b>37.2</b>	8.0	7.4	<b>9.3</b>	30.9	23.4	<b>32.1</b>
<b>Total nitrogen stocks</b>															
1500	0.6	0.5	<b>-27.2</b>	0.7	0.6	<b>-23.9</b>	0.8	0.6	<b>-23.2</b>	0.8	0.4	<b>-53.5</b>	3.0	2.0	<b>-32.6</b>
900	2.3	4.2	<b>81.0</b>	3.1	2.4	<b>-20.3</b>	2.8	2.6	<b>-7.1</b>	3.1	3.6	<b>16.3</b>	11.3	12.8	<b>13.6</b>
700	4.1	3.7	<b>-10.9</b>	3.1	3.3	<b>7.1</b>	3.8	4.5	<b>19.6</b>	3.8	4.6	<b>19.9</b>	14.8	16.1	<b>8.6</b>
500	1.3	1.4	<b>8.4</b>	2.1	1.7	<b>-17.1</b>	2.5	2.0	<b>-21.2</b>	2.6	2.1	<b>-19.0</b>	8.5	7.2	<b>-14.9</b>
350	0.4	0.5	<b>33.0</b>	0.6	0.6	<b>4.1</b>	0.8	0.8	<b>-2.5</b>	0.8	0.9	<b>6.0</b>	2.6	2.7	<b>6.7</b>
300	0.7	1.1	<b>47.1</b>	1.0	1.2	<b>18.5</b>	1.0	1.3	<b>36.6</b>	1.1	1.1	<b>6.7</b>	3.8	4.8	<b>25.2</b>



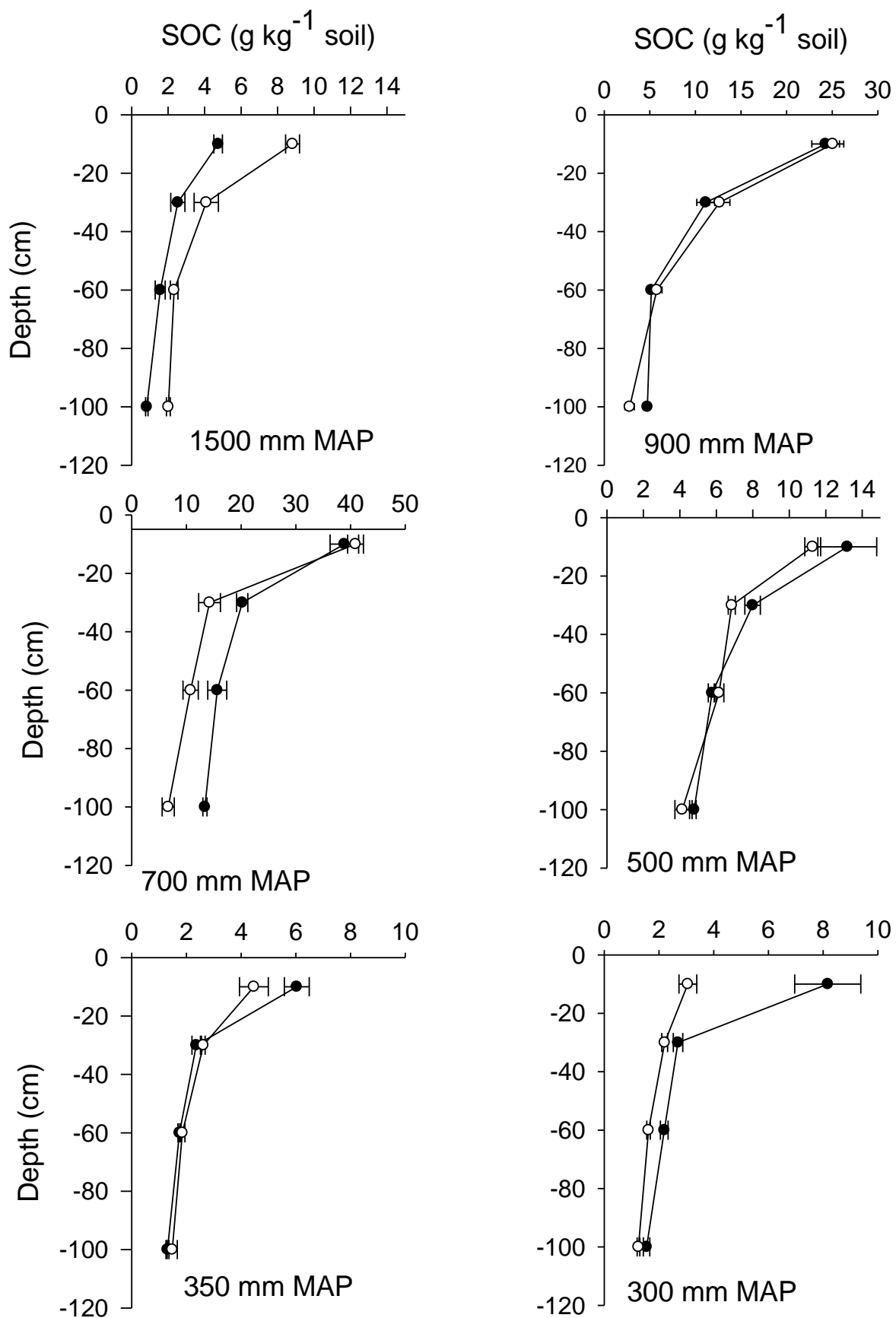


Figure 1

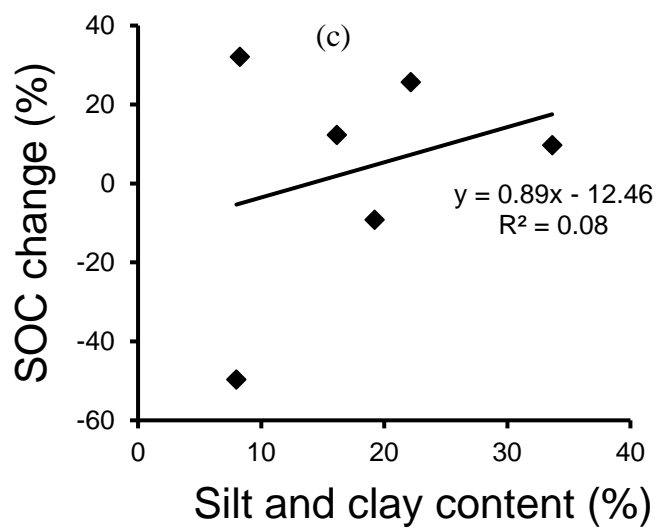
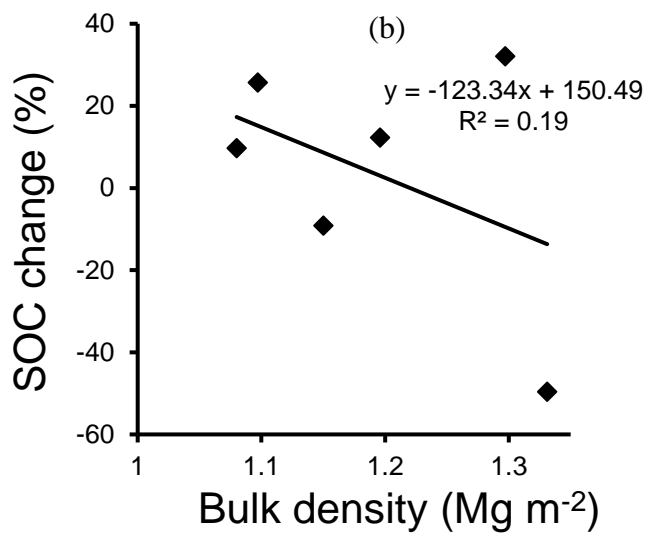
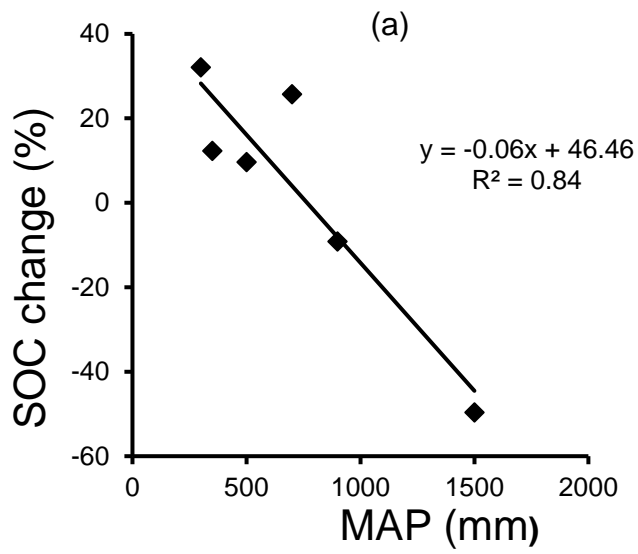


Figure 2

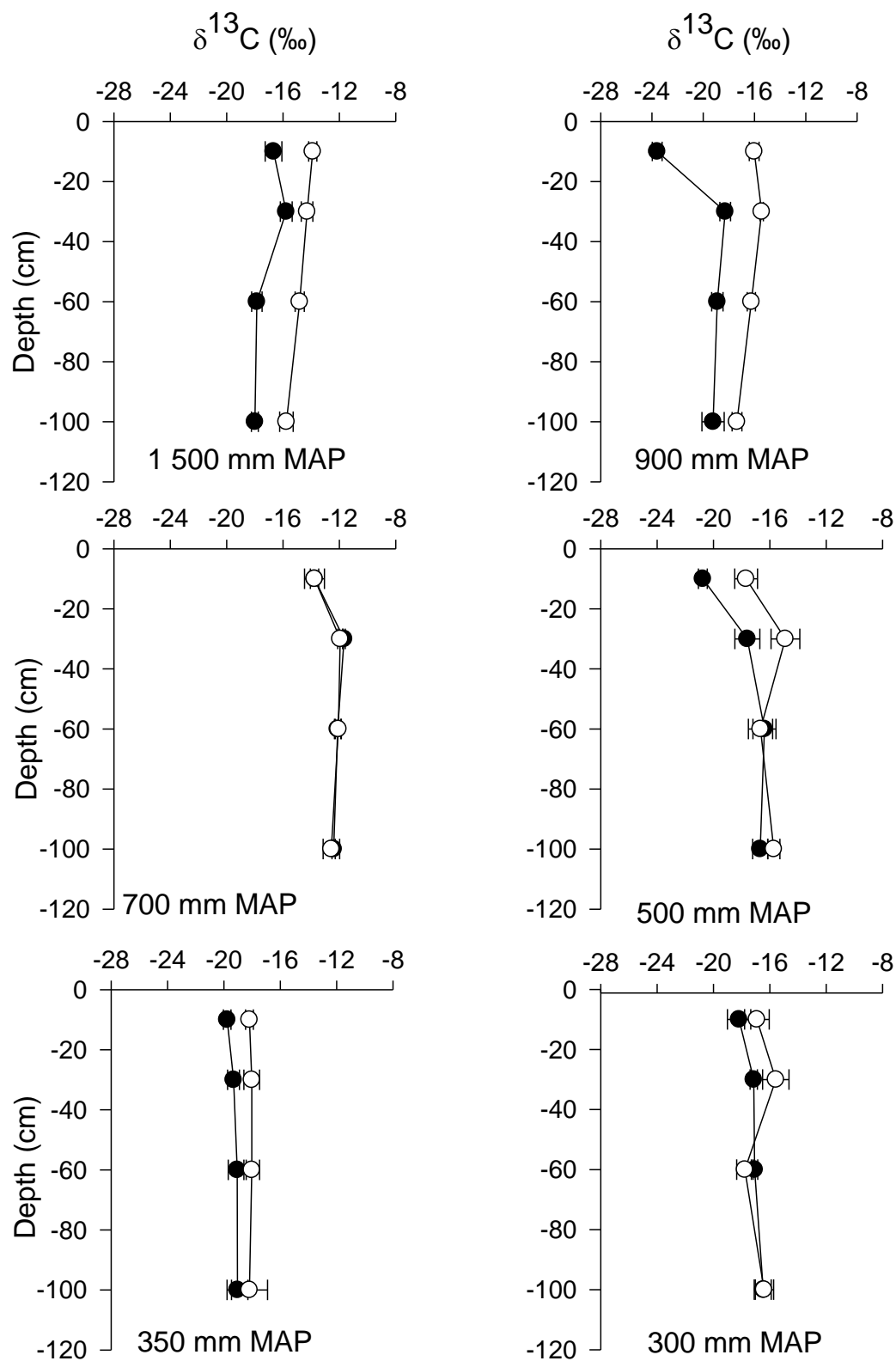
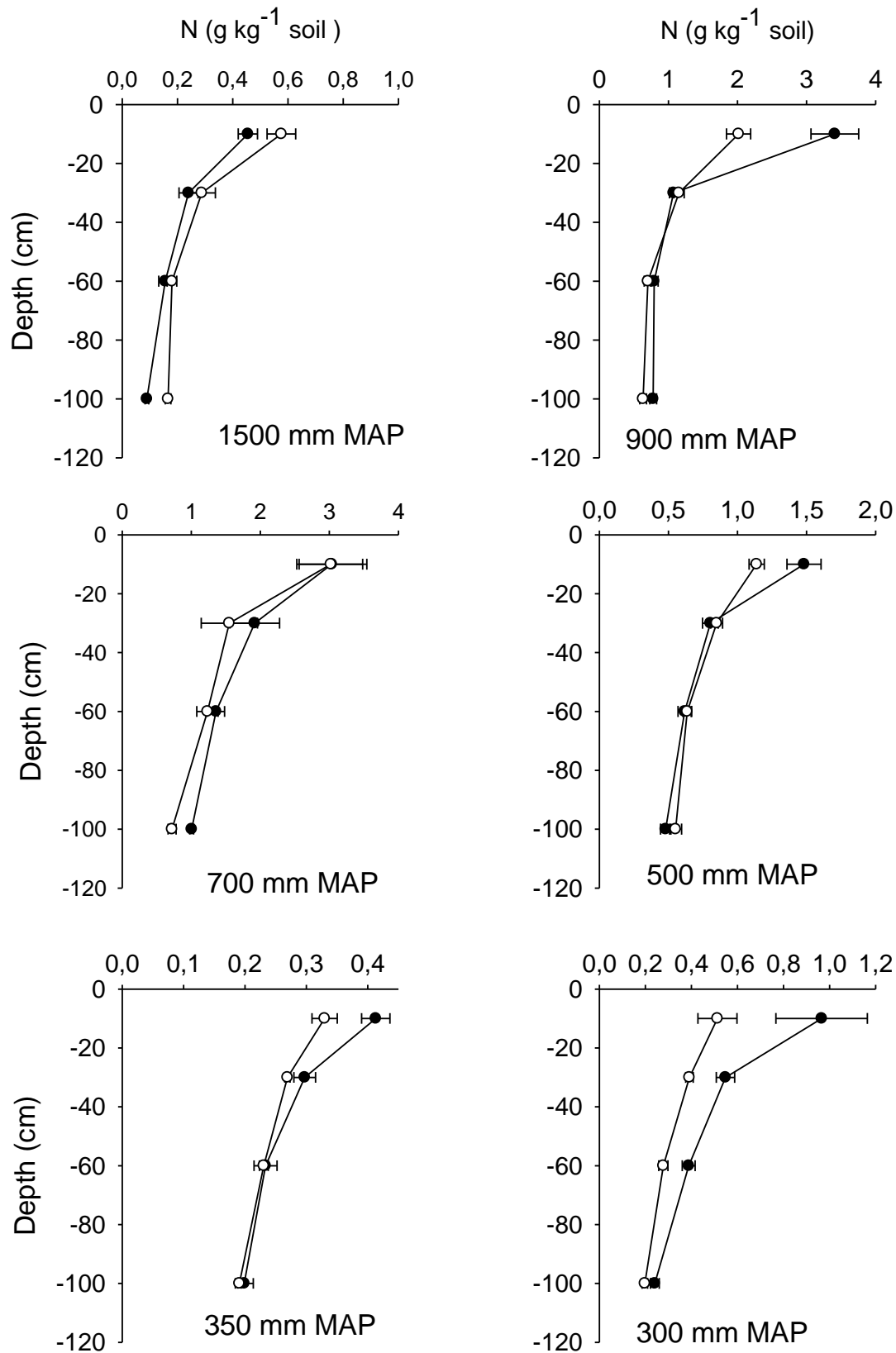


Figure 3

# 1 Supplementary material



2

3 Figure 4 Soil total-N concentrations (g N kg<sup>-1</sup> soil) with depth (cm) along a precipitation  
4 gradient (300-1500 mm MAP) in encroached grassland (●) and open grasslands (○).

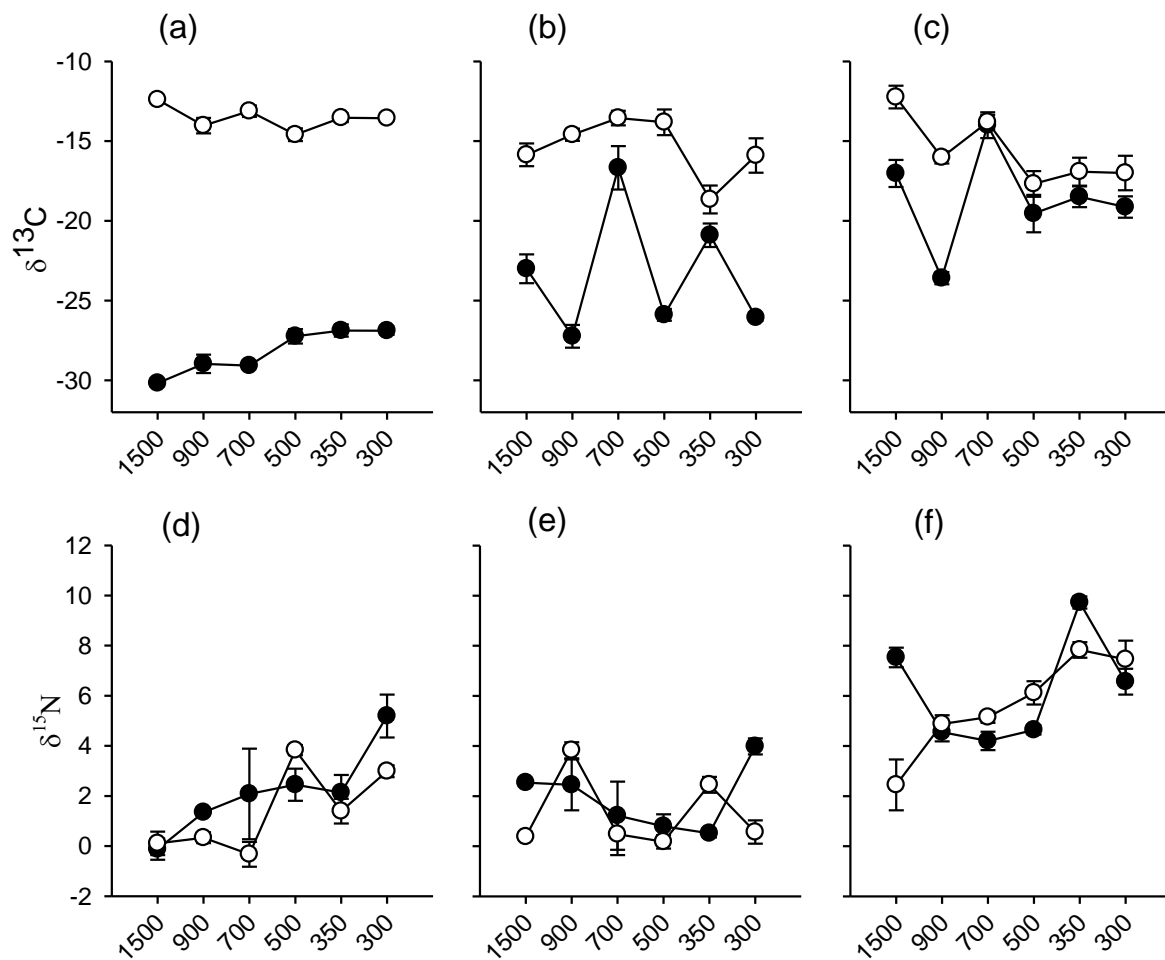
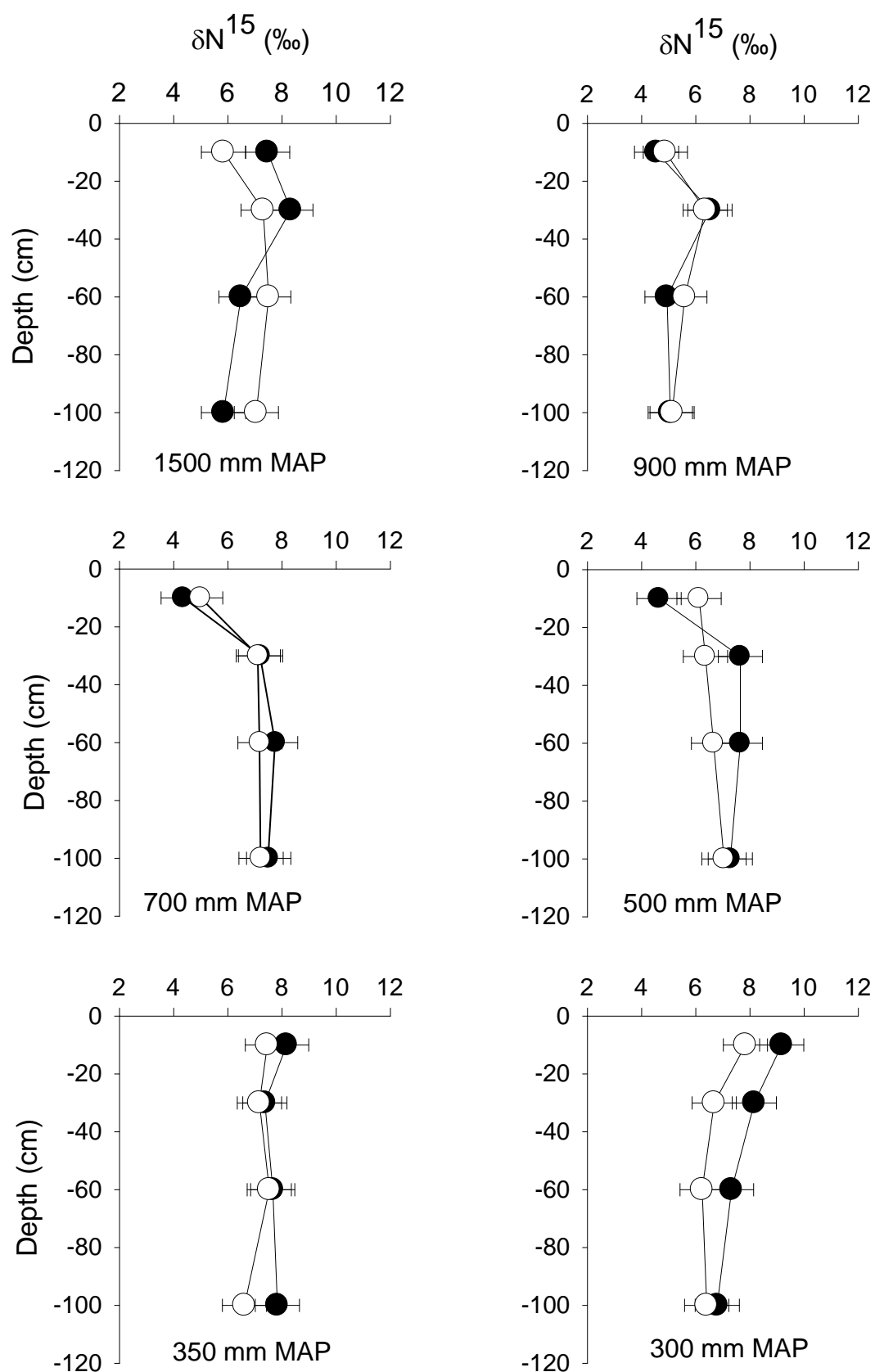


Figure 5  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in open grasslands (open circles) and encroached grasslands (black circles) in plant material (a and d), plant litter (b and e) and top soil (c and f). There is generally  $^{13}\text{C}$  enrichment from plant material to the top soil in the encroached grasslands relative to the corresponding open grasslands. There is also a general enrichment of  $\delta^{15}\text{N}$  from plant material to top soil in both the encroached and open grasslands.



1

2 Figure 6  $\delta N^{15}$  values of soil organic carbon in encroached grassland (●) and open grasslands  
3 (○) with depth (cm) in South Africa. Error bars are standard errors of the mean. The reference  
4 standard was atmospheric nitrogen.

## CHAPTER 4: CARBON AND NITROGEN IN SOIL PHYSICAL FRACTIONS FOLLOWING WOODY PLANT INVASION OF GRASSLAND

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This chapter has been formatted for *Plant and Soil*. A.M. conceived the study presented, collected and analysed the data and wrote the paper. PC and DW contributed substantially to experimental design, data analysis and manuscript preparation.

## 1   **ABSTRACT**

2  
3   Woody plant encroachment has extensively transformed land-cover patterns in savannas and  
4   grasslands throughout the world, with implications for changes in soil organic carbon (SOC)  
5   dynamics. However, our understanding of the nature of the effects of woody encroachment is  
6   very limited. In a study of pairs of neighbouring, woody encroached and grassland plots  
7   along a rainfall gradient (300 mm to 1500 mm per mean annual precipitation), we quantified  
8   the changes in carbon (C) and nitrogen (N) pools to a depth of 100 cm, in three soil physical  
9   fractions (coarse particulate organic matter, macroaggregates and silt and clay) and in bulk  
10   soil. In the 0-10 cm soil layer of the 1500 mm site, SOC stocks under shrub encroachment  
11   was 26 % lower than in open grassland with a total loss of 20 % of bulk soil C at 0-100 cm  
12   depths due to shrub encroachment. At the intermediate-rainfall sites of 900, 700 and  
13   500 mm MAP, there were no differences in the bulk soil C in encroached sites and open  
14   grasslands in the 0-10 cm soil layer. However, C in all the soil fractions at these sites was  
15   greater in shrub-encroached areas than open grasslands. The 900, 700 and 500 mm MAP sites  
16   lost 0.94, 4.19 and 12.37 % in total bulk SOC at 0-10 cm depth due to shrub encroachment,  
17   respectively. In the 0-100 cm depths, the 900 mm MAP site lost 13 % while 700, 500, 350  
18   and 300 mm MAP sites gained 14, 12, 24 and 27 % total SOC, respectively, due to shrub  
19   encroachment. In the soil fractions, the largest changes, ranging from 2.64 – 464 %, were in  
20   the coarse particulate organic matter (cPOM) and was lowest in the silt and clay fraction  
21   (6-32 %) across all sites and depth. Soil nitrogen generally increased across all encroached  
22   sites compared to open grasslands, likely due to the presence of nitrogen-fixing plants. These  
23   findings indicate that shrub-encroached grasslands potentially sequester more carbon in soils  
24   of arid regions than in humid regions.



## 1    **Introduction**

2  
3    Soils are a major reservoir of terrestrial carbon (C), storing more C (2344 Gt C up to 3 m  
4    depth) than aboveground terrestrial biomass (560 Gt C) and atmospheric pools (750 Gt C)  
5    combined (Amundson 2001). The annual C dioxide flux from soil to the atmosphere can be  
6    10 times greater than that from the combustion of fossil fuels in degraded and cultivated  
7    lands (Schlesinger 1997). This implies that a small change in soil organic C (SOC) can have a  
8    great impact on atmospheric C and subsequently on earth's climate. Land-cover change is  
9    one of the main factors that can alter SOC content (Edmondson et al. 2014).

10            Shrub encroachment in grassland and savanna biomes may currently be altering the  
11    global C and nitrogen (N) cycle in terrestrial ecosystems (Goodale and Davidson 2002). The  
12    invasion of shrubs in grasslands is generally thought to lead to an increase in the amount of C  
13    and N stored in these ecosystems (i.e. they are carbon sinks) because of the associated  
14    increase in net primary productivity (Hibbard et al. 2001a, Norris et al. 2001, Pacala et al.  
15    2001, Barger et al. 2011). Woody plants create islands of fertility where SOC and N may  
16    accumulate due to changes in litter quality and quantity (Schlesinger et al. 1990, McCulley et  
17    al. 2004, Allington and Valone 2014). Canopies of woody plants reduce the intensity of solar  
18    radiation and soil surface temperature, leading to reduced subcanopy decomposition, which  
19    may lead to an increase in SOC and N (Throop and Archer 2007). Jackson et al. (2002) found  
20    that there was a greater increase in SOC stored in the woody encroached sites than in the  
21    grasslands in drier locations (ca. ~400 mm MAP). They also observed SOC decrease in  
22    encroached sites unlike in grassland in the wetter locations. In a meta-analysis of available C  
23    data on shrub encroachment from North America, Barger et al. (2011) found soil organic C  
24    response to shrub encroachment was highly variable and unrelated to MAP. Barger et al.  
25    (2011) reported that SOC accumulated linearly ( $r^2 = 0.76$ ) with increasing clay content. They

1 also found that soil C accumulation decreased with increasing woody species encroachment  
2 as soil bulk density increased ( $r^2 = 0.52$ ) with carbon losses being confined to soil densities  
3 above 1.6. A number of studies have also reported a change in net N mineralisation and  
4 nitrification rates and a change to soil total N stocks due to shrub encroachment (Hibbard et  
5 al. 2001b, Archer et al. 2004, McCulley et al. 2004).

6 One challenge to understanding the effects of shrub encroachment on SOC and N is  
7 that the majority of studies in shrub-encroached grassland have focused on bulk soil C and N  
8 rather than C and N contained in different soil aggregates (Throop et al. 2013). The physical  
9 association of soil C and N, e.g. with respect to soil pores and aggregates, causes differential  
10 accessibility to decomposers and results in a range of SOC and N pools that differ in stability  
11 and dynamics (Throop et al. 2013).

12 Soil organic matter can be physically separated into coarse particulate organic matter  
13 (cPOM), microaggregate-protected organic matter ( $M_{agg}$ ) and silt and clay fraction (Stewart  
14 et al. 2009). Coarse particulate organic matter (cPOM) which consists of unprotected organic  
15 matter ( $>250\mu m$ ) is not intimately associated with soil mineral particles and is not  
16 occluded within aggregates. cPOM includes rapidly metabolised plant and associated  
17 microbial carbohydrates and more recalcitrant molecules derived from resistant plant  
18 materials and microbial decomposition products (Six et al. 2001). Microaggregate-  
19 protected organic matter is physically protected within 53-250  $\mu m$ -sized aggregates while the  
20 silt and clay fraction is  $<53 \mu m$  in size. SOC and N in the clay and silt fraction is more stable  
21 mainly because this soil fraction contains occluding organic materials, making them  
22 inaccessible to degrading organisms and their enzymes (Six et al. 2002, Stewart et al. 2009).

23 Although the physical fractionation of SOC and N has been widely employed in the  
24 study of land cover/ land-use effects in agricultural systems (Post and Kwon 2000, Stewart et

al. 2009), application of these techniques to the study of SOC and N in natural systems is less frequent (Throop et al. 2013). The objective of the study was to evaluate the effects of shrub encroachment in grasslands on SOC content across a rainfall gradient. We predict that SOC and N would be higher in encroached grasslands relative to unencroached grasslands in low-rainfall areas while there will be no association in high rainfall areas (Jackson et al. 2002). The reason behind our prediction is that humid grasslands are often highly productive, allocating a large proportion of C belowground with high root turnover, and have high SOC concentrations compared to dry grasslands (Jackson et al. 2002). We also predict that C will accumulate in cPOM under shrubs in drier areas because of slower decomposition (Liao and Boutton 2008), whereas in the more humid regions SOC may be greater in the silt and clay because C is more decomposed.

## **Materials and methods**

### **Study sites**

The study was carried out in six sites across a rainfall gradient in South Africa. The sites were KwaMbonambi (1500 mm MAP), Stanger (900 mm MAP), Bergville (700 mm MAP), Bloemfontein (500 mm MAP), Pniel (350 mm MAP) and Middelburg (300 mm MAP). The study site descriptions are summarised in Table 1.

### **Soil sampling**

Soil samples were collected between October 2013 and March 2014. At each site, 12 10 m x 10 m plots (six in grass-dominated (=open) and six in shrub-encroached grasslands) were randomly selected. To minimise differences among the land-cover types, plots with comparable elevation, aspect and soil type were selected at each site.

In each plot, a 1 m-deep pit was dug. After excluding recognisable soil surface litter, two soil samples at each depth interval of 0-10, 10-30, 30-60 and 60-100 cm were collected using stainless steel cores (6 cm in diameter by 10 cm in height). One sample from each depth was air dried at 105 °C to constant weight for the determination of bulk density. The other sample at each depth was air dried and passed through a 2000 µm sieve prior to fractionation.

## **Soil physical fractionation**

## **Soil carbon and nitrogen analysis**

The soil fractions and the bulk soil samples were analysed for C and N. Soil organic C was analysed using the loss-on-ignition method while N was analysed using an Elementar® Rapid N Cube analyser (Hanau, Germany). The SOC and N stocks were calculated using the following equation:

$$S_s = x_1 x_2 x_3 \quad (1)$$

where  $S_s$  is the SOC or nitrogen stocks ( $\text{Mg C ha}^{-1}$ );  $x_1$  is the C or N concentration in the soil material (%);  $x_2$  is the soil bulk density ( $\text{Mg m}^{-3}$ );  $x_3$  is the thickness of the soil layer (cm).

Data were tested for normality and homogeneity of variance using a Kolmogorov-Smirnoff test and Levene's test, respectively. The data on C and N concentration and stocks (mass) in soil fractions and bulk soil were analysed using a multivariate ANOVA analysis (MANOVA) to reduce problems with Type I error because we examined several dependent variables simultaneously. Wilks' Lambda was used as the multivariate measure. Carbon and N were the dependent variables whilst site (in terms of MAP), land-cover type (encroached and open grassland), soil depth and soil fractions were fixed factors.

## Results

The most humid site (KwaMbonambi, 1500 mm MAP) in the 0-10 and 10-30 cm layers had significantly higher bulk SOC in the open ( $F_{1,6}=111.55$ ,  $P < 0.01$ ;  $F_{1,6}=6.122$ ,  $P = 0.08$ ) than shrub-encroached grasslands (Table 3). The 0-10 cm layer had higher C in all soil fractions (cPOM;  $F_{1,6}=13.066$ ,  $P = 0.01$ ,  $M_{agg}$ ;  $F_{1,6}=14.401$ ,  $P = 0.009$ , silt and clay;  $F_{1,6}=95.808$ ,  $P < 0.001$ ) in open grasslands compared to shrub-encroached grasslands. At this 1500 mm MAP site in the 0-10 cm layer, shrub-encroached grassland lost 26 % of total soil carbon stocks. This loss was mainly driven by carbon loss in the cPOM and  $M_{agg}$  fraction, which lost 18 % and 17 % carbon, respectively. The accumulative loss to 100 cm depth at this site due to shrub encroachment was 20 %. Bulk soil nitrogen in the 0-10 cm layer at this 1500 mm MAP site was also significantly higher ( $F_{1,6} = 1057.83$ ,  $P < 0.001$ ) in the encroached than in the open grasslands (Table 5). The soil physical fractions at this site had generally higher nitrogen in the encroached grassland than in open grasslands (Table 5). The cPOM and  $M_{agg}$  recorded the highest percentage change in nitrogen due to shrub encroachment (Table 5).

At the 900 mm (Stanger), 700 mm (Bergville) and 500 mm (Bloemfontein) MAP sites, soil bulk C was not significantly different between encroached and open grassland sites in all four soil layers (Table 3). However, there were some significant differences for the soil physical fraction SOC at all three sites. At the 900 mm MAP site, in the 0-10 cm soil layer, cPOM and  $M_{agg}$  fractions C were significantly higher in the shrub-encroached grassland ( $F_{1,6} = 7.665$ ,  $P = 0.033$  and  $F_{1,6} = 10.77$ ,  $P = 0.17$ , respectively) compared to the open grassland. At Bergville (700 mm MAP), cPOM and  $M_{agg}$  C were significantly higher ( $F_{1,6} = 27.88$ ,  $P = 0.002$ ;  $F_{1,6}=32.65$ ,  $P = 0.001$ ) in the 10-30 cm soil layer in the encroached grassland than in open grassland. In Bloemfontein (500 mm MAP), at 0-10 cm depth,  $M_{agg}$  C was significant lower ( $F_{1,6} = 8.44$ ,  $P = 0.027$ ) while the silt and clay fraction C was higher ( $F_{1,6} = 50.69$ ,  $P < 0.001$ ) in encroached compared to open grasslands. Intermediate sites (900,

700 and 500 mm MAP) also lost 0.94 %, 4.19 % and 12.37 %, respectively, of total soil carbon at 0-10 cm depth. At the 900 mm MAP site in the 0-100 cm soil layer there was a gain of 2.53 % bulk-soil C due to shrub encroachment. Although the 700 and 500 mm MAP sites lost C in the encroached grassland relative to open grassland in the upper soil layers, these sites gained at greater depths, which resulted in them gaining 14.34 % and 11.76 % C, respectively, over the 0-100 cm depths. At these three mesic sites, N was generally higher in both the bulk soil and physical fractions across all soil layers (Table 4).

In the semi-arid sites of 350 mm MAP (Pniel) and 300 mm MAP (Middelburg), bulk soil organic C concentration in the 0-10 cm soil layer was significantly higher in the encroached grassland (350 mm MAP:  $F_{1,6} = 124.47$ ,  $P < 0.001$ ; 300 mm MAP:  $F_{1,6} = 50.11$ ,  $P < 0.001$ ) than in the open grassland. A similar trend of higher soil bulk C in the encroached grassland compared to open grassland was also shown at 30-60 cm depth and also at 60-100 cm depth in the semi-arid sites (Table 2).

The 350 mm MAP site (Pniel) had significantly higher cPOM,  $M_{agg}$  and silt and clay C ( $F_{1,6} = 289.34$ ,  $P < 0.001$ ;  $F_{1,6} = 383.30$ ,  $P < 0.01$ ;  $F_{1,6} = 124.47$ ,  $P < 0.001$ ) in the shrub-encroached grassland compared to the open grassland at 0-10 cm depth. This 350 mm MAP site, at 0-10 cm depth, gained 50 % of bulk soil C. In the 0-10 cm soil layer of the 300 mm MAP site (Middelburg), cPOM and  $M_{agg}$  C were significantly higher ( $F_{1,6} = 41.7$ ,  $P < 0.01$ ;  $F_{1,6} = 124.53$ ,  $P < 0.001$ ) in shrub-encroached grassland relative to the open grassland. The 300 mm MAP site, at 0-10 cm depth, gained 50 % of bulk soil C. The gain in the two drier sites was mostly driven by accumulation of carbon in the cPOM fraction, which gained 108 % and 112 %, respectively. These sites (350 and 300 mm MAP) mostly gained carbon across all depths relative to open grassland. At 0-100 cm, the encroached grassland in these two drier sites gained 27 % and 24 % of bulk soil carbon relative to open grassland.

Soil nitrogen in the semi-arid sites was generally higher in the encroached grasslands compared to open grasslands across all depths.

The C:N ratio at 0-10 and 10-30 cm depths for 1500, 900 and 500 mm MAP sites was less in the encroached grasslands compared to the open grasslands (Fig 1a, 1b and 1c). However, for the drier sites (350 and 300 mm MAP), the C:N ratio was greater in the encroached than in the open grasslands (Fig 1e and 1f).

There was a significant negative correlation between C change in cPOM and MAP ( $r = -0.87$ ,  $P = 0.03$ ) (Fig 2a). Although the correlations between C change due to shrub encroachment in both  $M_{agg}$  and silt + clay fractions and MAP were negative, the relationships were not significant ( $r = -0.78$ ,  $P = 0.7$ ;  $r = -0.65$ ,  $P = 0.16$ , respectively) (Fig 2b and 2c).

## Discussion

The findings of this study are in agreement with Geesing et al. (2000), Jackson et al. (2002), and Wheeler et al. (2007) who found a negative correlation between change in SOC between encroached and open grassland and MAP. Our results, however, were contrary to the review done by Barger et al. (2011) who found that C response to shrub encroachment was highly variable and unrelated to MAP. The increase of SOC in the drier sites is in agreement with the well-described island of fertility (Liao et al. 2006, Allington and Valone 2014), where shrubs and trees develop extensive root systems that track nutrients from the surroundings, leading to local nutrient recycling and organic matter accumulation (Belsky et al. 1989). The greater accumulation of SOC in the shrub-encroached grasslands than in open grasslands could also be due to greater rates of primary productivity in shrubs than in grasses. Greater primary productivity increases organic matter input in the soil beneath deep-rooted shrubs

1 through increased surface litter and accumulation of complex organic compounds, which  
2 are resistant to decay (Liao et al. 2006).

3         The soil fraction showed that the increase in SOC in the dry sites was driven by the  
4 accumulation of C in the coarse particulate organic matter (cPOM) and microaggregate-  
5 protected organic matter ( $M_{agg}$ ) fractions. In these fractions in the dry sites, SOC was almost  
6 double in the encroached grassland than in the open grassland. The cPOM fraction contains  
7 unprotected C which consists of plant debris and entrained mineral matter easily lost back  
8 into the atmosphere (Six et al. 2002). Other studies have shown that under tree and shrub  
9 canopies there was reduced solar irradiance (45-65 %), reduced soil temperatures (5-11 °C),  
10 and lower rainfall (0-50%) (Belsky et al. 1989, Eldridge et al. 2011). The decrease in solar  
11 radiation and soil temperature may result in reduced C mineralisation in the cPOM fraction,  
12 allowing the carbon to have longer residence time prior to incorporation into the physically  
13 protected  $M_{agg}$  fraction (Liao and Boutton 2008). The silt and clay fraction was generally  
14 unchanged, especially in the 0-10 cm soil layer, because carbon may need several decades to  
15 hundreds of years to be incorporated into this fraction (Six et al. 2002, Stewart et al. 2009).  
16 This finding is contrary to the review of Barger et al. (2011) who found a positive  
17 relationship between clay content and soil C change with encroachment. Our findings  
18 indicate that the encroachment at our sites did not occur long enough in the past to cause  
19 change in the silt and clay fraction in the upper soil layers. The proposal that woody plant  
20 encroachment could be fairly recent in our drier site is supported by Ward et al. (2014) who  
21 found out that the rate of encroachment has increased substantially in recent times. However,  
22 in the deeper soil layers, silt and clay C was significantly higher in the shrub-encroached  
23 compared to the open (unencroached) grassland. This perhaps indicates that it requires less  
24 time for carbon to be incorporated into the more stable aggregates at greater depths than it  
25 requires at the surface.



Our findings in the 500, 700 and 900 mm MAP sites are contrary to the findings of Jackson et al. (2002) who found that from >500 mm annual of rainfall there will generally be loss of SOC due to woody species encroachment. These sites which had mean annual rainfall ranging from 500 – 900 mm showed no significant differences in bulk soil C concentration in the encroached site relative to the open grassland in the 0-10 cm soil layer. The cPOM and  $M_{agg}$  fraction in these mesic sites, however, generally showed that they had more C in the encroached grasslands compared to the open (unencroached) grasslands across all depths. The effects of the fractions may have been too small to significantly change the overall bulk soil C in the 0-10 cm layer of the encroached sites.

The 1500 mm MAP site had more C in the open grassland compared to the encroached grassland. This is consistent with the findings of Jackson et al. (2002). In high-rainfall areas, grasslands are highly productive and allocate a large proportion of SOC below ground (Jackson et al. 2002).

#### **Soil nitrogen**

Soil nitrogen was mostly high in the encroached grassland across all sites at all depths, probably because the encroaching woody species are mostly nitrogen-fixing plants. Nitrogen-fixing plants ameliorate and increase nitrogen in the soil (Blaser et al. 2013). This finding is consistent with results from other African savanna ecosystems, where sites encroached by woody species have been compared with open grasslands (Ludwig et al. 2004, Kambatuku et al. 2013, Blaser et al. 2014). However, we note that the driest site, Middelburg, is not encroached by legumes (*Searsia erosa*, *S. burchellii*, *Diospyros lycioides* and *Eriocephalus ericoides*) and yet still shows this effect. The difference in soil nitrogen between the encroached and the unencroached sites seem to have been driven by the cPOM and  $M_{agg}$  fraction nitrogen.

## **C:N ratios**

The results showed a lower C:N ratio in the open (unencroached) grasslands compared to the encroached grasslands in the moister sites (500, 700, 900 and 1500 mm MAP) while in the semi-arid sites (350 and 300 mm MAP) the ratio was higher in the open grasslands. The higher C: N ratios in the arid sites indicate that there was a lower proportional increase of nitrogen compared to carbon. One of the factors that might have caused the disproportionate increase of nitrogen in the arid sites could be high soil temperatures, low soil moisture content and the soil type (Walsh 1995). High soil temperatures limit nodulation (Hungria and Vargas 2000) and, hence, reduce nitrogen fixation. Low soil moisture, as is experienced in 350 and 300 mm MAP sites, also compromises plant rhizobial growth, and is a major cause of nodulation failure and low nitrogen fixation (Durand et al. 1987, Walsh 1995). The low nitrogen levels in the semi-arid site could also be due to severe stress which may also lead to irreversible cessation of nitrogen fixation (Hungria and Vargas 2000).

## **Conclusions**

This study generally showed that soil organic carbon increases in low rainfall areas while it decreases in very high rainfall areas due to shrub encroachment. Soil nitrogen increased across all sites due to shrub encroachment. The change in SOC and N was mainly driven by C accumulation/loss in the cPOM fraction. We were able to distinguish the differences between encroached sites and unencroached sites in soil fractions C but were unable to do so with regard to soil bulk C in our 900, 700 and 500 mm MAP sites. Thus, carbon in the soil fractions may be a better parameter to use in trying to understand the effects of shrub encroachment in savanna and grassland ecosystems.

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Table 1: Study site descriptions according to Mucina and Rutherford (2010). GPS coordinates refer to the coordinates of one of the corners of the site.\* indicate nitrogen-fixing species.

Site	GPS coordinates	Annual Rainfall (mm)	Temperature (°C) Min      Max		Biome	Soil Characteristics	Major plant species
KwaMbonambi	28° 49' 32" S 32° 16' 96.92"E	1500	3.5	35	Maputaland wooded grasslands	Quaternary redistributed sands supporting yellowish redistributed sands of the Berea formation	<i>Sporobolus fimbriatus</i> , <i>Digitaria natalensis</i> (grasses); <i>Diospyros lycioides</i> * (shrub); <i>Terminalia sericea</i> (tree)
Stanger	29° 18' 59.05" S 31° 22' 13.24"E	900	5.8	32.6	KwaZulu-Natal Coastal Belt	Ordovician Natal group sandstone	<i>Themeda triandra</i> , <i>Aristida</i> (grasses), <i>A. karroo</i> * (tree)
Bergville	28° 79' 06.30" S 29° 38' 98.40"E	700	5.8	32.6	KwaZulu-Natal moist grasslands	Ordovician Natal group sandstone	<i>Themeda triandra</i> , <i>Hyparrhenia hirta</i> (grasses); <i>Acacia karroo</i> *, <i>A sieberiana</i> * (trees)
Bloemfontein	28° 59' 16.77" S 26° 16' 54.24"E	450	0	32	Bloemfontein dry grasslands	Sedimentary mudstones and layers of sandstone	<i>Aristida congesta</i> , <i>Aristida diffusa</i> , <i>Cynodon dactylon</i> (grasses); <i>Acacia karroo</i> * (tree)
Pniel	28° 34' 50.00" S 24° 30' 30.70"E	350	-4.1	37.5	Kimberley thornveld	Sandy to loam soils of the Hutton soil form	<i>Eragrotis curvula</i> , <i>Schmidtia pappophoroides</i> (grasses); <i>Acacia erioloba</i> *, <i>A tortilis</i> *, <i>A. karroo</i> *, <i>A. mellifera</i> *, <i>Tarchonanthus camphoratus</i> (trees)
Middelburg	31° 25' 24" S 24° 58' 82.10"E	300	-7.2	36.1	Eastern Upper Karoo	Sandy to loam soils of the Hutton soil form	<i>Aristida</i> , <i>Eragrostis</i> (grasses); <i>Searsia erosa</i> , <i>S. burchellii</i> , <i>Diospyros lycioides</i> * and <i>Eriocephalus ericoides</i> (shrubs)

Table 2: Soil organic carbon (SOC) concentrations (mean  $\pm$  standard error (g C kg<sup>-1</sup> soil)) in bulk soil and three soil fractions; coarse particulate organic matter (cPOM), microaggregates M<sub>agg</sub>, and silt and clay fractions for four depths across six sites along a rainfall gradient in a shrub-encroached grassland (“Shrub”) compared to adjacent open grassland (“Grass”). The table shows that soil physical fraction predicts effects of shrub encroachment on SOC better than to bulk soils. Bold numbers indicate significant differences at P = 0.05 between encroached and open grassland. The 0-100 cm depth SOC concentrations are mean values of 0-10, 10-30, 30-60 and 60-100 cm depths.

Site	Depth (cm)	Bulk soil		Coarse particulate organic matter		Microaggregates		Silt and clay	
		Shrub	Grass	Shrub	Grass	Shrub	Grass	Shrub	Grass
KwaMbonambi (1500 mm MAP)	0-10	<b>12.82 <math>\pm</math> 0.26</b>	<b>15.89 <math>\pm</math> 0.26</b>	<b>2.67 <math>\pm</math> 0.07</b>	<b>3.01 <math>\pm</math> 0.07</b>	<b>7.86 <math>\pm</math> 0.16</b>	<b>8.73 <math>\pm</math> 0.16</b>	<b>2.29 <math>\pm</math> 0.14</b>	<b>4.16 <math>\pm</math> 0.14</b>
	10-30	<b>10.53 <math>\pm</math> 0.32</b>	<b>11.66 <math>\pm</math> 0.32</b>	<b>2.55 <math>\pm</math> 0.08</b>	<b>2.39 <math>\pm</math> 0.08</b>	<b>6.11 <math>\pm</math> 0.21</b>	<b>6.76 <math>\pm</math> 0.20</b>	<b>1.88 <math>\pm</math> 0.05</b>	<b>2.52 <math>\pm</math> 0.11</b>
	30-60	8.80 $\pm$ 0.36	9.69 $\pm$ 0.36	2.06 $\pm$ 0.07	1.92 $\pm$ 0.07	<b>5.34 <math>\pm</math> 0.15</b>	<b>5.83 <math>\pm</math> 0.15</b>	<b>1.41 <math>\pm</math> 0.15</b>	<b>1.95 <math>\pm</math> 0.15</b>
	60-100	6.94 $\pm$ 0.31	7.45 $\pm$ 0.31	1.71 $\pm$ 0.13	1.53 $\pm$ 0.13	4.29 $\pm$ 0.21	4.52 $\pm$ 0.21	<b>0.94 <math>\pm</math> 0.07</b>	<b>1.40 <math>\pm</math> 0.07</b>
	0-100	8.81 $\pm$ 0.31	9.80 $\pm$ 0.31	2.08 $\pm$ 0.07	1.97 $\pm$ 0.06	5.32 $\pm$ 0.17	5.77 $\pm$ 0.17	<b>1.40 <math>\pm</math> 0.09</b>	<b>2.07 <math>\pm</math> 0.09</b>
Stanger (900 mm MAP)	0-10	8.65 $\pm$ 0.26	8.04 $\pm$ 0.26	<b>2.69 <math>\pm</math> 0.07</b>	<b>2.41 <math>\pm</math> 0.07</b>	<b>3.56 <math>\pm</math> 0.14</b>	<b>2.92 <math>\pm</math> 0.14</b>	2.42 $\pm$ 0.21	2.71 $\pm$ 0.21
	10-30	4.94 $\pm$ 0.18	5.14 $\pm$ 0.18	<b>1.76 <math>\pm</math> 0.11</b>	<b>1.28 <math>\pm</math> 0.11</b>	<b>1.83 <math>\pm</math> 0.06</b>	<b>1.93 <math>\pm</math> 0.06</b>	<b>1.35 <math>\pm</math> 0.08</b>	<b>1.94 <math>\pm</math> 0.08</b>
	30-60	3.63 $\pm$ 0.21	3.69 $\pm$ 0.21	<b>1.28 <math>\pm</math> 0.10</b>	<b>0.89 <math>\pm</math> 0.10</b>	1.33 $\pm$ 0.10	1.28 $\pm$ 0.10	<b>1.10 <math>\pm</math> 0.04</b>	<b>1.51 <math>\pm</math> 0.04</b>
	60-100	2.53 $\pm$ 0.12	2.71 $\pm$ 0.12	<b>0.85 <math>\pm</math> 0.05</b>	<b>0.58 <math>\pm</math> 0.05</b>	<b>0.86 <math>\pm</math> 0.03</b>	<b>1.00 <math>\pm</math> 0.09</b>	<b>0.81 <math>\pm</math> 0.01</b>	<b>1.13 <math>\pm</math> 0.01</b>
	0-100	3.99 $\pm$ 0.15	4.02 $\pm$ 0.29	<b>1.35 <math>\pm</math> 0.07</b>	<b>1.00 <math>\pm</math> 0.07</b>	1.46 $\pm$ 0.05	1.47 $\pm$ 0.05	<b>1.14 <math>\pm</math> 0.08</b>	<b>1.56 <math>\pm</math> 0.08</b>
Bergville (700 mm MAP)	0-10	45.27 $\pm$ 2.33	39.48 $\pm$ 2.32	14.46 $\pm$ 1.33	12.89 $\pm$ 1.33	20.12 $\pm$ 1.55	18.04 $\pm$ 1.55	10.69 $\pm$ 0.91	8.56 $\pm$ 0.91
	10-30	<b>27.77 <math>\pm</math> 1.5</b>	<b>18.67 <math>\pm</math> 1.50</b>	<b>9.63 <math>\pm</math> 1.74</b>	<b>4.93 <math>\pm</math> 0.57</b>	<b>12.65 <math>\pm</math> 0.53</b>	<b>7.33 <math>\pm</math> 0.53</b>	5.47 $\pm$ 0.64	6.39 $\pm$ 0.64
	30-60	22.88 $\pm$ 1.82	19.21 $\pm$ 1.81	<b>7.14 <math>\pm</math> 0.23</b>	<b>4.83 <math>\pm</math> 0.23</b>	<b>10.78 <math>\pm</math> 0.94</b>	<b>7.18 <math>\pm</math> 0.94</b>	<b>4.95 <math>\pm</math> 0.96</b>	<b>7.19 <math>\pm</math> 0.96</b>
	60-100	16.60 $\pm$ 1.65	14.65 $\pm$ 1.65	<b>4.93 <math>\pm</math> 0.31</b>	<b>2.80 <math>\pm</math> 0.31</b>	5.74 $\pm$ 0.78	6.06 $\pm$ 0.78	5.96 $\pm$ 0.70	5.77 $\pm$ 0.70
	0-100	<b>23.58 <math>\pm</math> 1.34</b>	<b>19.30 <math>\pm</math> 1.34</b>	<b>7.47 <math>\pm</math> 0.40</b>	<b>4.85 <math>\pm</math> 0.40</b>	<b>10.07 <math>\pm</math> 0.62</b>	<b>7.85 <math>\pm</math> 0.62</b>	6.03 $\pm$ 0.52	6.60 $\pm$ 0.52
Bloemfontein (500 mm MAP)	0-10	9.01 $\pm$ 1.14	7.35 $\pm$ 0.77	<b>1.57 <math>\pm</math> 0.37</b>	<b>1.09 <math>\pm</math> 0.26</b>	<b>3.06 <math>\pm</math> 0.133</b>	<b>3.34 <math>\pm</math> 0.15</b>	<b>4.46 <math>\pm</math> 0.61</b>	<b>2.94 <math>\pm</math> 0.34</b>
	10-30	6.26 $\pm$ 0.45	5.58 $\pm$ 0.29	0.53 $\pm$ 0.85	0.56 $\pm$ 0.20	<b>2.45 <math>\pm</math> 0.20</b>	<b>2.67 <math>\pm</math> 0.95</b>	<b>3.28 <math>\pm</math> 0.16</b>	<b>2.35 <math>\pm</math> 0.52</b>
	30-60	4.40 $\pm$ 0.37	4.53 $\pm$ 0.37	0.11 $\pm$ 0.06	0.13 $\pm$ 0.09	<b>1.98 <math>\pm</math> 0.16</b>	<b>1.26 <math>\pm</math> 0.20</b>	2.31 $\pm$ 0.16	2.13 $\pm$ 0.22
	60-100	<b>3.30 <math>\pm</math> 0.50</b>	<b>2.68 <math>\pm</math> 1.18</b>	<b>0.31 <math>\pm</math> 0.06</b>	<b>0.053 <math>\pm</math> 0.06</b>	<b>1.43 <math>\pm</math> 0.34</b>	<b>1.76 <math>\pm</math> 0.21</b>	1.56 $\pm$ 0.12	0.87 $\pm$ 0.09
	0-100	4.79 $\pm$ 0.41	4.28 $\pm$ 0.28	<b>0.42 <math>\pm</math> 0.04</b>	<b>0.30 <math>\pm</math> 0.03</b>	<b>1.96 <math>\pm</math> 0.06</b>	<b>1.95 <math>\pm</math> 0.11</b>	<b>2.42 <math>\pm</math> 0.32</b>	<b>1.75 <math>\pm</math> 0.29</b>
Pniel (350 mm MAP)	0-10	<b>2.11 <math>\pm</math> 0.09</b>	<b>1.62 <math>\pm</math> 0.10</b>	0.14 $\pm$ 0.02	0.14 $\pm$ 0.02	<b>0.96 <math>\pm</math> 0.02</b>	<b>0.53 <math>\pm</math> 0.02</b>	1.01 $\pm$ 0.06	0.96 $\pm$ 0.06
	10-30	1.37 $\pm$ 0.08	1.25 $\pm$ 0.03	<b>1.11 <math>\pm</math> 0.03</b>	<b>0.93 <math>\pm</math> 0.03</b>	<b>0.71 <math>\pm</math> 0.03</b>	<b>0.46 <math>\pm</math> 0.04</b>	<b>0.79 <math>\pm</math> 0.03</b>	<b>0.56 <math>\pm</math> 0.03</b>
	30-60	1.12 $\pm$ 0.04	1.11 $\pm$ 0.04	0.10 $\pm$ 0.03	0.08 $\pm$ 0.03	0.51 $\pm$ 0.00	0.42 $\pm$ 0.00	0.51 $\pm$ 0.03	0.41 $\pm$ 0.27
	60-100	<b>0.65 <math>\pm</math> 0.46</b>	<b>0.44 <math>\pm</math> 0.1</b>	0.73 $\pm$ 0.04	0.09 $\pm$ 0.06	0.35 $\pm$ 0.45	0.20 $\pm$ 0.02	0.51 $\pm$ 0.02	0.54 $\pm$ 0.03
	0-100	<b>1.58 <math>\pm</math> 0.11</b>	<b>1.39 <math>\pm</math> 0.11</b>	<b>0.56 <math>\pm</math> 0.01</b>	<b>0.26 <math>\pm</math> 0.01</b>	<b>0.53 <math>\pm</math> 0.01</b>	<b>0.35 <math>\pm</math> 0.02</b>	0.66 $\pm$ 0.05	0.54 $\pm$ 0.05
Middelburg (300 mm MAP)	0-10	<b>4.17 <math>\pm</math> 0.07</b>	<b>2.7 <math>\pm</math> 0.07</b>	<b>0.91 <math>\pm</math> 0.02</b>	<b>0.34 <math>\pm</math> 0.02</b>	<b>2.07 <math>\pm</math> 0.05</b>	<b>1.26 <math>\pm</math> 0.05</b>	1.2 $\pm$ 0.04	1.09 $\pm$ 0.04
	10-30	<b>2.93 <math>\pm</math> 0.06</b>	<b>2.14 <math>\pm</math> 0.07</b>	<b>0.54 <math>\pm</math> 0.01</b>	<b>0.28 <math>\pm</math> 0.01</b>	<b>1.38 <math>\pm</math> 0.04</b>	<b>1.08 <math>\pm</math> 0.04</b>	<b>1.02 <math>\pm</math> 0.03</b>	<b>0.78 <math>\pm</math> 0.03</b>
	30-60	<b>2.45 <math>\pm</math> 0.06</b>	<b>1.76 <math>\pm</math> 0.06</b>	<b>0.46 <math>\pm</math> 0.01</b>	<b>0.22 <math>\pm</math> 0.01</b>	<b>1.12 <math>\pm</math> 0.04</b>	<b>0.93 <math>\pm</math> 0.03</b>	<b>0.87 <math>\pm</math> 0.02</b>	<b>0.61 <math>\pm</math> 0.02</b>
	60-100	<b>2.03 <math>\pm</math> 0.11</b>	<b>1.36 <math>\pm</math> 0.11</b>	<b>0.36 <math>\pm</math> 0.02</b>	<b>0.17 <math>\pm</math> 0.02</b>	<b>0.92 <math>\pm</math> 0.06</b>	<b>0.71 <math>\pm</math> 0.05</b>	<b>0.75 <math>\pm</math> 0.04</b>	<b>0.48 <math>\pm</math> 0.04</b>
	0-100	<b>2.44 <math>\pm</math> 0.11</b>	<b>1.77 <math>\pm</math> 0.11</b>	<b>0.47 <math>\pm</math> 0.02</b>	<b>0.22 <math>\pm</math> 0.02</b>	<b>1.16 <math>\pm</math> 0.04</b>	<b>0.91 <math>\pm</math> 0.04</b>	<b>0.81 <math>\pm</math> 0.06</b>	<b>0.64 <math>\pm</math> 0.06</b>



Table 3: Soil organic carbon stocks (Mg/ha) of bulk soil (B.soil) and three soil fractions: coarse particulate organic matter (cPOM), macroaggregates ( $M_{agg}$ ), and silt and clay fractions for four depths across six sites along a rainfall gradient under shrub-encroached grasslands (“Shrub”) compared to adjacent open grasslands (“Grass”). % change = ((shrub-grass)/grass)\*100. The table shows that there was a general loss of carbon due to shrub encroachment in areas with high mean annual precipitation (KwaMbonambi and Stanger), while shrub encroachment resulted in more soil carbon in semi-arid regions (Pniel and Middelburg). Bold numbers indicate significant differences between encroached and open grassland.

Site	Soil fractions	0-10 cm			0-30 cm			30-60 cm			60-100 cm			0-100 cm		
		Shrub	Grass	% change	Shrub	Grass	% change	Shrub	Grass	% change	Shrub	Grass	% change	Shrub	Grass	% change
KwaMbonambi (1500 mm MAP)	B.soil	13.29	17.87	<b>-25.63</b>	24.77	30.05	<b>-17.57</b>	43.91	54.43	<b>-19.33</b>	44.29	55.03	<b>-19.52</b>	100.52	125.43	<b>-19.77</b>
	cPOM	2.78	3.39	<b>-17.99</b>	6.00	6.16	<b>-2.60</b>	10.28	10.79	<b>-4.73</b>	10.91	11.27	<b>-3.19</b>	23.76	25.17	<b>-5.19</b>
	$M_{agg}$	8.14	9.81	<b>-17.02</b>	14.36	17.4	<b>-17.47</b>	26.62	32.69	<b>-18.57</b>	27.35	33.38	<b>-18.06</b>	60.7	73.98	<b>-18.02</b>
	Silt+clay	4.37	4.68	<b>-6.62</b>	4.41	6.49	<b>-32.05</b>	7.01	10.94	<b>-35.92</b>	6.02	10.37	<b>-41.95</b>	25.05	26.29	<b>-32.85</b>
Stanger (900 mm MAP)	B.soil	10.55	9.15	<b>-0.94</b>	11.2	13.66	<b>-18.01</b>	15.89	11.20	<b>41.88</b>	17.40	19.67	<b>-11.54</b>	45.27	52.29	<b>2.53</b>
	cPOM	3.27	2.74	<b>19.34</b>	3.99	3.4	<b>17.35</b>	5.63	3.98	<b>41.46</b>	5.91	4.23	<b>39.72</b>	15.43	12.56	<b>31.01</b>
	$M_{agg}$	4.33	3.32	<b>0.23</b>	4.15	5.12	<b>-18.95</b>	5.86	4.16	<b>40.87</b>	5.89	7.27	<b>-18.98</b>	17.61	19.48	<b>1.81</b>
	Silt+clay	2.95	3.09	<b>-4.53</b>	3.06	5.14	<b>-40.47</b>	4.39	3.05	<b>43.93</b>	5.59	8.16	<b>-31.50</b>	17.04	19.74	<b>-17.75</b>
Bergville (750 mm MAP)	B.soil	46.54	47.03	<b>-4.19</b>	56.26	46.19	<b>21.80</b>	108.66	91.95	<b>18.17</b>	115.87	119.73	<b>-3.22</b>	244.16	213.53	<b>7.36</b>
	cPOM	14.87	15.35	<b>-14.69</b>	19.54	12.21	<b>60.03</b>	33.92	23.13	<b>46.65</b>	34.20	23.09	<b>48.12</b>	82.66	63.26	<b>38.97</b>
	$M_{agg}$	20.68	21.48	<b>-3.72</b>	25.63	18.15	<b>41.21</b>	51.21	34.36	<b>49.04</b>	40.08	49.51	<b>-19.05</b>	111.43	98.41	<b>11.42</b>
	Silt+clay	10.99	10.18	<b>7.96</b>	11.09	15.82	<b>-29.90</b>	23.52	34.47	<b>-31.77</b>	41.59	47.12	<b>-11.74</b>	55.941	59.71	<b>-18.96</b>
Bloemfontein (500 mm MAP)	B.soil	2.62	2.99	<b>-12.37</b>	2.95	3.93	<b>-24.94</b>	3.43	4.34	<b>-20.97</b>	9.91	5.66	<b>75.09</b>	18.91	16.92	<b>11.76</b>
	cPOM	1.9	1.23	<b>54.47</b>	1.2	1.49	<b>-19.46</b>	0.36	0.53	<b>-32.08</b>	1.41	0.25	<b>464.00</b>	4.87	3.5	<b>39.14</b>
	$M_{agg}$	1.31	2.27	<b>-42.29</b>	1.49	1.98	<b>-24.75</b>	1.83	2.43	<b>-24.69</b>	6.23	1.72	<b>262.21</b>	10.86	8.4	<b>29.29</b>
	Silt+clay	0.86	0.65	<b>32.31</b>	1.16	1.15	<b>0.87</b>	1.49	1.54	<b>-3.25</b>	3.3	3.05	<b>8.20</b>	6.81	6.39	<b>6.57</b>
Pniel (350 mm MAP)	B.soil	2.73	1.82	<b>50.00</b>	3.56	2.84	<b>25.35</b>	5.73	4.22	<b>35.78</b>	7.07	6.48	<b>9.10</b>	10.13	8.16	<b>24.28</b>
	cPOM	0.18	0.16	<b>20.00</b>	0.27	0.21	<b>28.57</b>	0.48	0.37	<b>29.73</b>	0.59	0.37	<b>59.46</b>	1.21	0.96	<b>36.94</b>
	$M_{agg}$	1.25	0.60	<b>108.33</b>	1.85	1.05	<b>76.19</b>	2.57	1.99	<b>29.15</b>	2.62	1.98	<b>32.32</b>	6.44	3.87	<b>47.51</b>
	Silt+clay	1.31	1.06	<b>3.97</b>	1.44	1.59	<b>-9.43</b>	2.67	1.87	<b>42.78</b>	4.84	4.02	<b>20.40</b>	5.43	5.13	<b>20.14</b>
Middelburg (300 mm MAP)	B.soil	4.18	3.43	<b>50.00</b>	7.20	5.88	<b>22.45</b>	12.27	8.48	<b>44.69</b>	13.65	12.06	<b>13.18</b>	10.13	8.16	<b>24.96</b>
	cPOM	0.91	0.43	<b>111.63</b>	1.29	0.76	<b>69.74</b>	2.31	1.05	<b>120.00</b>	2.62	1.51	<b>73.51</b>	5.69	3.49	<b>90.13</b>
	$M_{agg}$	2.08	1.61	<b>29.19</b>	3.39	2.98	<b>13.76</b>	5.60	4.47	<b>25.28</b>	6.64	6.29	<b>5.56</b>	14.1	12.14	<b>15.37</b>
	Silt+clay	1.19	1.39	<b>8.33</b>	2.52	2.14	<b>17.76</b>	4.36	2.94	<b>48.30</b>	4.39	4.25	<b>3.29</b>	10.79	10.1	<b>16.23</b>





Table 4: Soil nitrogen concentrations (mean  $\pm$  standard error (g N kg<sup>-1</sup> soil)) in Bulk Soil, cPOM, M<sub>agg</sub> and Silt + Clay in different soil fractions for four depths across all six sites along a rainfall gradient under shrub-encroached grasslands (“Shrub”) compared to adjacent open grasslands (“Grass”). Bold numbers indicate values that are significantly different at P= 0.05 between encroached and open grassland.

Site	Depth (cm)	Bulk soil		cPom		Microaggregate		Silt + clay	
		Shrub	Grass	Shrub	Grass	Shrub	Grass	Shrub	Grass
<b>KwaMbonambi</b> (1500 mm MAP)	0-10	1.45 $\pm$ 0.03	1.19 $\pm$ 0.03	<b>0.24<math>\pm</math>0.01</b>	<b>0.23<math>\pm</math>0.01</b>	<b>0.95<math>\pm</math>0.02</b>	<b>0.65<math>\pm</math>0.02</b>	0.28 $\pm$ 0.06	0.3 $\pm$ 0.06
	10-30	<b>1.14<math>\pm</math>0.04</b>	<b>0.89<math>\pm</math>0.06</b>	<b>0.26<math>\pm</math>0.01</b>	<b>0.18<math>\pm</math>0.00</b>	<b>0.70<math>\pm</math>0.03</b>	<b>0.54<math>\pm</math>0.03</b>	<b>0.19<math>\pm</math>0.00</b>	<b>0.18<math>\pm</math>0.04</b>
	30-60	<b>0.94<math>\pm</math>0.05</b>	<b>0.72<math>\pm</math>0.05</b>	0.23 $\pm$ 0.02	0.15 $\pm$ 0.02	<b>0.58<math>\pm</math>0.03</b>	<b>0.43<math>\pm</math>0.03</b>	<b>0.14<math>\pm</math>0.01</b>	<b>0.15<math>\pm</math>0.01</b>
	60-100	0.64 $\pm$ 0.03	0.56 $\pm$ 0.03	<b>0.17<math>\pm</math>0.01</b>	<b>0.11<math>\pm</math>0.01</b>	0.39 $\pm$ 0.03	0.34 $\pm$ 0.03	<b>0.08<math>\pm</math>0.01</b>	<b>0.11<math>\pm</math>0.00</b>
	0-100	<b>0.91<math>\pm</math>0.04</b>	<b>0.74<math>\pm</math>0.04</b>	<b>0.21<math>\pm</math>0.01</b>	<b>0.15<math>\pm</math>0.01</b>	<b>0.56<math>\pm</math>0.03</b>	<b>0.44<math>\pm</math>0.03</b>	<b>0.14<math>\pm</math>0.01</b>	<b>0.15<math>\pm</math>0.01</b>
<b>Stanger</b> (900 mm MAP)	0-10	<b>0.88<math>\pm</math>0.05</b>	<b>0.67<math>\pm</math>0.03</b>	<b>0.31<math>\pm</math>0.01</b>	<b>0.22<math>\pm</math>0.02</b>	<b>0.35<math>\pm</math>0.02</b>	<b>0.24<math>\pm</math>0.02</b>	0.22 $\pm$ 0.01	0.21 $\pm$ 0.01
	10-30	<b>0.50<math>\pm</math>0.02</b>	<b>0.40<math>\pm</math>0.02</b>	<b>0.17<math>\pm</math>0.02</b>	<b>0.10<math>\pm</math>0.02</b>	<b>0.18<math>\pm</math>0.02</b>	<b>0.15<math>\pm</math>0.00</b>	0.14 $\pm$ 0.01	0.15 $\pm$ 0.00
	30-60	0.34 $\pm$ 0.02	<b>0.28<math>\pm</math>0.02</b>	<b>0.11<math>\pm</math>0.01</b>	0.07 $\pm$ 0.01	0.12 $\pm$ 0.00	0.11 $\pm$ 0.00	0.10 $\pm$ 0.00	0.11 $\pm$ 0.00
	60-100	<b>0.26<math>\pm</math>0.00</b>	<b>0.23<math>\pm</math>0.02</b>	<b>0.07<math>\pm</math>0.00</b>	<b>0.04<math>\pm</math>0.00</b>	0.10 $\pm$ 0.00	0.09 $\pm$ 0.00	0.09 $\pm$ 0.00	0.09 $\pm$ 0.00
	0-100	<b>0.39<math>\pm</math>0.01</b>	<b>0.32<math>\pm</math>0.01</b>	<b>0.13<math>\pm</math>0.01</b>	<b>0.08<math>\pm</math>0.01</b>	<b>0.15<math>\pm</math>0.00</b>	<b>0.12<math>\pm</math>0.00</b>	0.12 $\pm$ 0.01	0.12 $\pm$ 0.03
<b>Bergville</b> (700 mm MAP)	0-10	3.46 $\pm$ 0.20	3.28 $\pm$ 0.09	<b>1.02<math>\pm</math>0.01</b>	<b>1.06<math>\pm</math>0.01</b>	1.51 $\pm$ 0.06	1.44 $\pm$ 0.04	0.89 $\pm$ 0.06	0.78 $\pm$ 0.02
	10-30	<b>2.11<math>\pm</math>0.02</b>	<b>1.53<math>\pm</math>0.01</b>	<b>0.70<math>\pm</math>0.16</b>	<b>0.38<math>\pm</math>0.09</b>	<b>0.99<math>\pm</math>0.09</b>	<b>0.55<math>\pm</math>0.06</b>	0.42 $\pm$ 0.08	0.60 $\pm$ 0.02
	30-60	1.70 $\pm$ 0.09	1.61 $\pm$ 0.35	<b>0.47<math>\pm</math>0.02</b>	<b>0.38<math>\pm</math>0.03</b>	<b>0.86<math>\pm</math>0.03</b>	<b>0.59<math>\pm</math>0.02</b>	<b>0.37<math>\pm</math>0.01</b>	<b>0.64<math>\pm</math>0.01</b>
	60-100	1.31 $\pm$ 0.12	1.18 $\pm$ 0.12	<b>0.38<math>\pm</math>0.02</b>	<b>0.16<math>\pm</math>0.02</b>	0.44 $\pm$ 0.09	0.48 $\pm$ 0.01	0.49 $\pm$ 0.08	0.52 $\pm$ 0.08
	0-100	<b>1.80<math>\pm</math>0.10</b>	<b>1.59<math>\pm</math>0.01</b>	<b>0.54<math>\pm</math>0.03</b>	<b>0.36<math>\pm</math>0.03</b>	<b>0.78<math>\pm</math>0.05</b>	<b>0.62<math>\pm</math>0.05</b>	<b>0.48<math>\pm</math>0.04</b>	<b>0.60<math>\pm</math>0.04</b>
<b>Bloemfontein</b> (500 mm MAP)	0-10	<b>1.02<math>\pm</math>0.06</b>	<b>0.36<math>\pm</math>0.04</b>	<b>0.18<math>\pm</math>0.01</b>	<b>0.13<math>\pm</math>0.02</b>	<b>0.12<math>\pm</math>0.04</b>	<b>0.19<math>\pm</math>0.03</b>	0.07 $\pm$ 0.01	0.05 $\pm$ 0.01
	10-30	0.18 $\pm$ 0.01	0.18 $\pm$ 0.02	0.07 $\pm$ 0.01	0.07 $\pm$ 0.00	0.07 $\pm$ 0.01	0.08 $\pm$ 0.01	0.05 $\pm$ 0.01	0.04 $\pm$ 0.00
	30-60	0.12 $\pm$ 0.01	0.12 $\pm$ 0.01	0.01 $\pm$ 0.00	0.02 $\pm$ 0.00	0.05 $\pm$ 0.02	0.03 $\pm$ 0.00	0.04 $\pm$ 0.00	0.04 $\pm$ 0.00
	60-100	<b>0.13<math>\pm</math>0.01</b>	<b>0.08<math>\pm</math>0.00</b>	<b>0.03<math>\pm</math>0.01</b>	<b>0.01<math>\pm</math>0.00</b>	0.04 $\pm$ 0.00	0.04 $\pm$ 0.00	<b>0.05<math>\pm</math>0.00</b>	<b>0.03<math>\pm</math>0.00</b>
	0-100	0.16 $\pm$ 0.01	0.14 $\pm$ 0.01	0.05 $\pm$ 0.00	0.03 $\pm$ 0.00	0.06 $\pm$ 0.00	0.07 $\pm$ 0.00	0.06 $\pm$ 0.00	0.03 $\pm$ 0.00
<b>Pniel</b> (350 mm MAP)	0-10	<b>0.2<math>\pm</math>0.03</b>	<b>0.13<math>\pm</math>0.00</b>	<b>0.02<math>\pm</math>0.00</b>	<b>0.01<math>\pm</math>0.00</b>	<b>0.07<math>\pm</math>0.00</b>	<b>0.04<math>\pm</math>0.00</b>	<b>0.12<math>\pm</math>0.03</b>	<b>0.07<math>\pm</math>0.00</b>
	10-30	<b>0.13<math>\pm</math>0.02</b>	<b>0.11<math>\pm</math>0.00</b>	<b>0.02<math>\pm</math>0.00</b>	<b>0.01<math>\pm</math>0.00</b>	<b>0.04<math>\pm</math>0.01</b>	<b>0.03<math>\pm</math>0.00</b>	0.04 $\pm$ 0.01	0.03 $\pm$ 0.01
	30-60	<b>0.10<math>\pm</math>0.01</b>	<b>0.06<math>\pm</math>0.00</b>	<b>0.04<math>\pm</math>0.00</b>	<b>0.03<math>\pm</math>0.00</b>	<b>0.04<math>\pm</math>0.00</b>	<b>0.03<math>\pm</math>0.00</b>	<b>0.05<math>\pm</math>0.01</b>	<b>0.02<math>\pm</math>0.00</b>
	60-100	<b>0.09<math>\pm</math>0.01</b>	<b>0.05<math>\pm</math>0.00</b>	<b>0.02<math>\pm</math>0.00</b>	<b>0.01<math>\pm</math>0.00</b>	0.03 $\pm$ 0.00	0.03 $\pm$ 0.01	<b>0.04<math>\pm</math>0.00</b>	<b>0.01<math>\pm</math>0.00</b>
	0-100	<b>0.11<math>\pm</math>0.01</b>	<b>0.07<math>\pm</math>0.00</b>	<b>0.03<math>\pm</math>0.00</b>	<b>0.02<math>\pm</math>0.00</b>	<b>0.04<math>\pm</math>0.00</b>	<b>0.03<math>\pm</math>0.00</b>	<b>0.05<math>\pm</math>0.00</b>	<b>0.02<math>\pm</math>0.00</b>
<b>Middelburg</b> 300 mm MAP)	0-10	<b>0.33<math>\pm</math>0.03</b>	<b>0.25<math>\pm</math>0.00</b>	<b>0.1<math>\pm</math>0.00</b>	<b>0.03<math>\pm</math>0.00</b>	<b>0.18<math>\pm</math>0.00</b>	<b>0.13<math>\pm</math>0.00</b>	0.1 $\pm$ 0.03	0.1 $\pm$ 0.00
	10-30	<b>0.30<math>\pm</math>0.00</b>	<b>0.18<math>\pm</math>0.00</b>	<b>0.06<math>\pm</math>0.00</b>	<b>0.03<math>\pm</math>0.01</b>	<b>0.14<math>\pm</math>0.00</b>	<b>0.10<math>\pm</math>0.01</b>	<b>0.1<math>\pm</math>0.00</b>	<b>0.06<math>\pm</math>0.00</b>
	30-60	<b>0.27<math>\pm</math>0.01</b>	<b>0.16<math>\pm</math>0.01</b>	<b>0.05<math>\pm</math>0.00</b>	<b>0.03<math>\pm</math>0.00</b>	<b>0.14<math>\pm</math>0.01</b>	<b>0.09<math>\pm</math>0.01</b>	<b>0.09<math>\pm</math>0.00</b>	<b>0.05<math>\pm</math>0.00</b>
	60-100	<b>0.22<math>\pm</math>0.03</b>	<b>0.14<math>\pm</math>0.01</b>	<b>0.04<math>\pm</math>0.01</b>	<b>0.02<math>\pm</math>0.00</b>	0.11 $\pm$ 0.02	0.09 $\pm$ 0.01	<b>0.08<math>\pm</math>0.01</b>	<b>0.04<math>\pm</math>0.00</b>
	0-100	<b>0.25<math>\pm</math>0.01</b>	<b>0.16<math>\pm</math>0.01</b>	<b>0.05<math>\pm</math>0.01</b>	<b>0.03<math>\pm</math>0.00</b>	<b>0.13<math>\pm</math>0.00</b>	<b>0.09<math>\pm</math>0.01</b>	<b>0.08<math>\pm</math>0.01</b>	<b>0.05<math>\pm</math>0.00</b>

Table 5: Soil fractions and total N stocks (Mg/ha) to 1 m depth and total changes in N pools in shrub-encroached grasslands (“Shrub”) compared to adjacent open grasslands (“Grass”). % change = % change = ((shrub-grass)/grass)\*100.the table shows that there was generally increase in soil nitrogen due to shrub encroachment. Bold numbers indicate % differences between open and encroached grasslands. Negative numbers indicate that grass values are higher than shrub values.

Site	Soil Fractions	0-10 cm depth			10-30 cm depth			30-60 cm depth			60-100 cm depth			0-100 cm depth		
		Shrub	Grass	% Change	Shrub	Grass	% Change	Shrub	Grass	% Change	Shrub	Grass	% Change	Shrub	Grass	% Change
<b>KwaMbonambi (1500 mm MAP)</b>	B.soil	1.49	1.34	<b>11.19</b>	2.70	2.29	<b>17.90</b>	4.71	4.01	<b>17.46</b>	4.06	4.15	<b>-2.17</b>	10.78	7.79	<b>38.38</b>
	cPOM	0.23	0.26	<b>-11.54</b>	0.61	0.47	<b>29.79</b>	1.15	0.82	<b>40.24</b>	1.05	0.83	<b>26.51</b>	2.32	1.30	<b>78.46</b>
	Magg	0.98	0.74	<b>32.43</b>	1.64	1.38	<b>18.84</b>	2.87	2.40	<b>19.58</b>	2.49	2.50	<b>-0.40</b>	6.86	4.76	<b>44.12</b>
	Silt+clay	0.28	0.34	<b>-17.65</b>	0.44	0.45	<b>-2.22</b>	0.69	0.82	<b>-15.85</b>	0.51	0.82	<b>-37.80</b>	1.57	1.70	<b>-7.65</b>
<b>Stanger (900 mm MAP)</b>	B.soil	1.07	0.76	<b>40.79</b>	1.13	1.06	<b>6.60</b>	1.49	1.51	<b>-1.32</b>	1.78	1.66	<b>7.23</b>	3.94	3.73	<b>5.63</b>
	cPOM	0.38	0.25	<b>52.00</b>	0.39	0.28	<b>39.29</b>	0.49	0.37	<b>32.43</b>	0.47	0.32	<b>46.88</b>	0.93	0.71	<b>30.99</b>
	Magg	0.43	0.27	<b>59.26</b>	0.41	0.39	<b>5.13</b>	0.55	0.56	<b>-1.79</b>	0.67	0.66	<b>1.52</b>	1.68	1.52	<b>10.53</b>
	Silt+clay	0.26	0.23	<b>13.04</b>	0.32	0.39	<b>-17.95</b>	0.45	0.57	<b>-21.05</b>	0.64	0.67	<b>-4.48</b>	1.34	1.49	<b>-10.07</b>
<b>Bergville (700 mm MAP)</b>	B.soil	3.56	3.91	<b>-8.95</b>	4.27	3.77	<b>13.26</b>	8.06	7.71	<b>4.54</b>	9.12	9.60	<b>-5.00</b>	24.75	19.30	<b>28.24</b>
	cPOM	1.11	1.27	<b>-12.60</b>	1.43	0.94	<b>52.13</b>	2.22	1.83	<b>21.31</b>	2.68	1.31	<b>104.58</b>	5.97	3.53	<b>69.12</b>
	Magg	1.56	1.71	<b>-8.77</b>	2.00	1.35	<b>48.15</b>	4.09	2.81	<b>45.55</b>	3.04	3.98	<b>-23.62</b>	8.66	7.70	<b>12.47</b>
	Silt+clay	0.89	0.93	<b>-4.30</b>	0.85	1.48	<b>-42.57</b>	1.75	3.07	<b>-43.00</b>	3.39	4.31	<b>-21.35</b>	6.30	6.98	<b>-9.74</b>
<b>Bloemfontein (500 mm MAP)</b>	B.soil	0.45	0.41	<b>9.76</b>	0.41	0.48	<b>-14.58</b>	0.48	0.61	<b>-21.31</b>	1.00	0.57	<b>75.44</b>	1.27	1.18	<b>7.63</b>
	cPOM	0.22	0.15	<b>46.67</b>	0.15	0.18	<b>-16.67</b>	0.05	0.10	<b>-50.00</b>	0.24	0.04	<b>500.00</b>	0.56	0.50	<b>12.00</b>
	Magg	0.14	0.21	<b>-33.33</b>	0.11	0.11	<b>0.00</b>	0.24	0.32	<b>-25.00</b>	0.47	0.21	<b>123.81</b>	1.13	0.73	<b>54.79</b>
	Silt+clay	0.09	0.05	<b>80.00</b>	0.11	0.11	<b>0.00</b>	0.48	0.61	<b>-21.31</b>	1.00	0.57	<b>75.44</b>	0.48	0.40	<b>20.00</b>
<b>Pniel (350 mm MAP)</b>	B.soil	0.26	0.14	<b>85.71</b>	0.34	0.25	<b>36.00</b>	0.32	0.44	<b>-27.27</b>	0.39	0.33	<b>18.18</b>	1.28	1.04	<b>23.08</b>
	cPOM	0.02	0.01	<b>100.00</b>	0.02	0.02	<b>0.00</b>	0.20	0.16	<b>25.00</b>	0.03	0.03	<b>0.00</b>	0.10	0.09	<b>11.11</b>
	Magg	0.16	0.08	<b>100.00</b>	0.15	0.09	<b>66.67</b>	0.15	0.08	<b>87.50</b>	0.15	0.12	<b>25.00</b>	0.53	0.33	<b>60.61</b>
	Silt+clay	0.20	0.07	<b>185.71</b>	0.17	0.14	<b>21.43</b>	0.06	0.18	<b>-66.67</b>	0.26	0.21	<b>23.81</b>	0.69	0.59	<b>16.95</b>
<b>Middelburg (300 mm MAP)</b>	B.soil	0.33	0.23	<b>43.48</b>	0.74	0.51	<b>45.10</b>	1.35	0.77	<b>75.32</b>	1.51	1.21	<b>24.79</b>	2.50	1.89	<b>32.28</b>
	cPOM	0.08	0.04	<b>100.00</b>	0.14	0.09	<b>55.56</b>	0.23	0.12	<b>91.67</b>	0.32	0.17	<b>88.24</b>	0.60	0.36	<b>66.67</b>
	Magg	0.18	0.16	<b>12.50</b>	0.36	0.26	<b>38.46</b>	0.70	0.42	<b>66.67</b>	0.74	0.73	<b>1.37</b>	1.63	1.26	<b>29.37</b>
	Silt+clay	0.07	0.03	<b>133.33</b>	0.24	0.16	<b>50.00</b>	0.43	0.22	<b>95.45</b>	0.44	0.31	<b>41.94</b>	0.30	0.23	<b>30.43</b>

## Figure Captions

**Figure 1:** C:N ratio of bulk soils and soil fractions (  bulk soil,  coarse particulate organic matter (cPOM);  macroaggregates ( $M_{agg}$ ),  silt and clay fraction, with depth in shrub-encroached grassland (“shrub”) and open grassland (“grass”) across a rainfall gradient.

**Figure 2:** Relationship between changes in SOC relative to shrub encroachment in three soil physical fractions at 0-100 cm depth with woody encroachment and mean annual precipitation (MAP) in mm (a) coarse particulate organic matter (cPOM); (b) macroaggregates ( $M_{agg}$ ) (c) silt and clay fraction. A positive value is achieved because there is more SOC in encroached soils than in open grassland soils and *vice versa*.

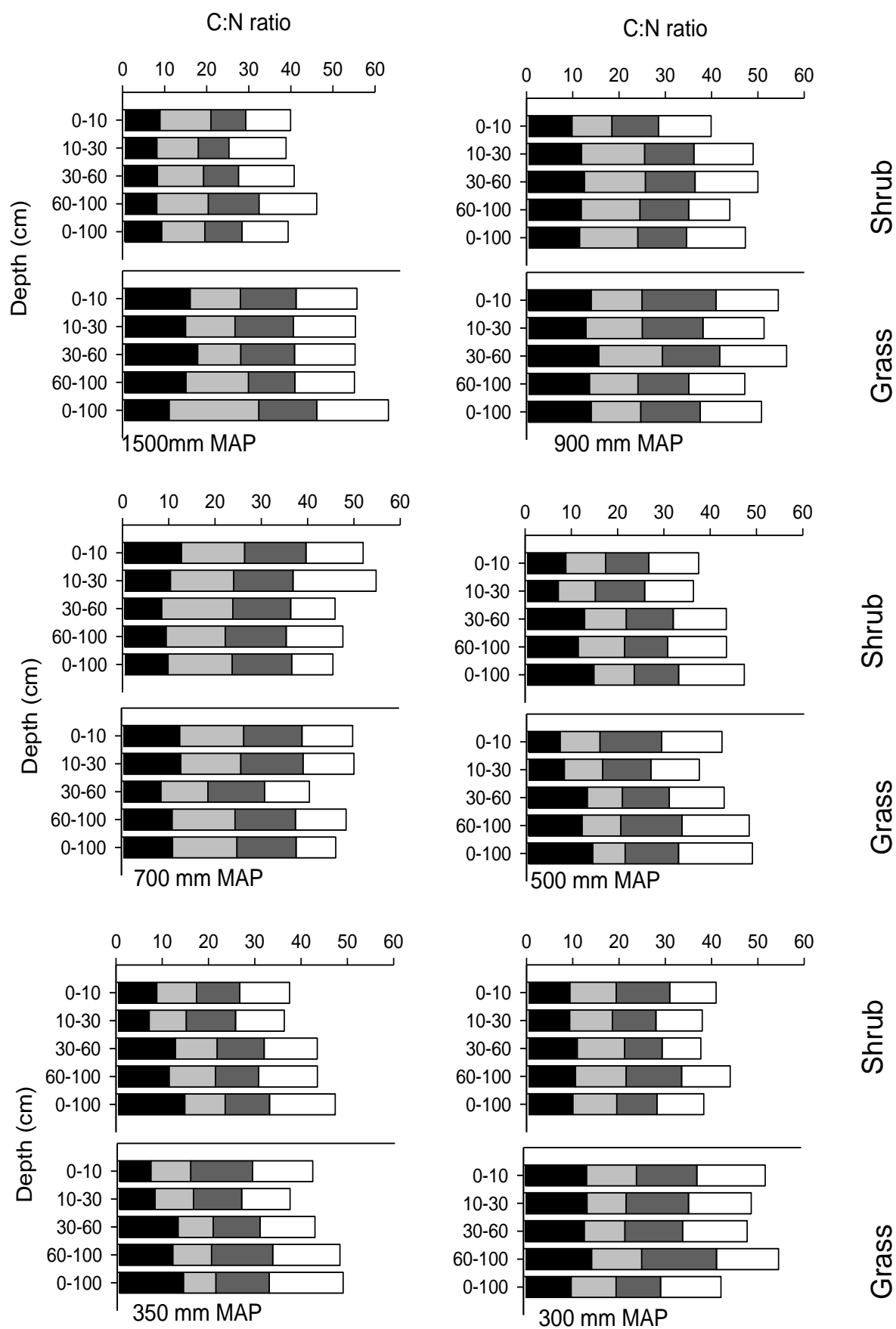
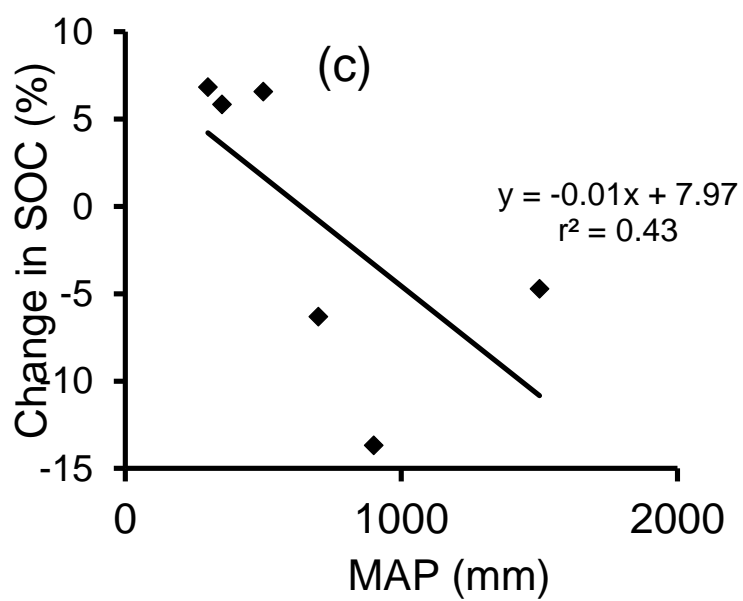
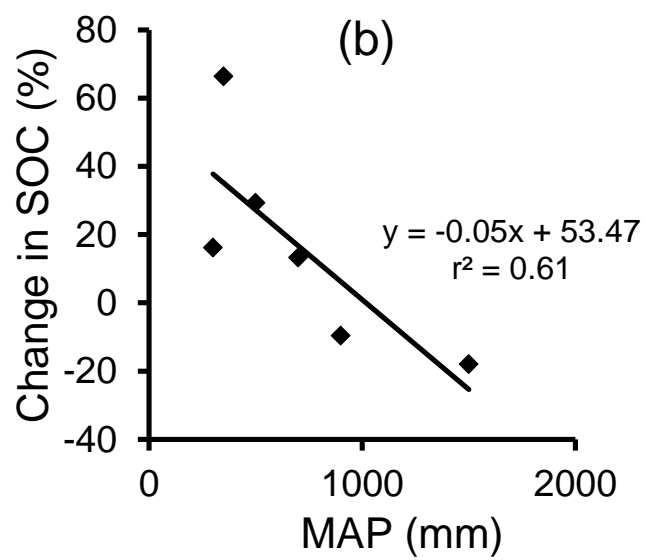
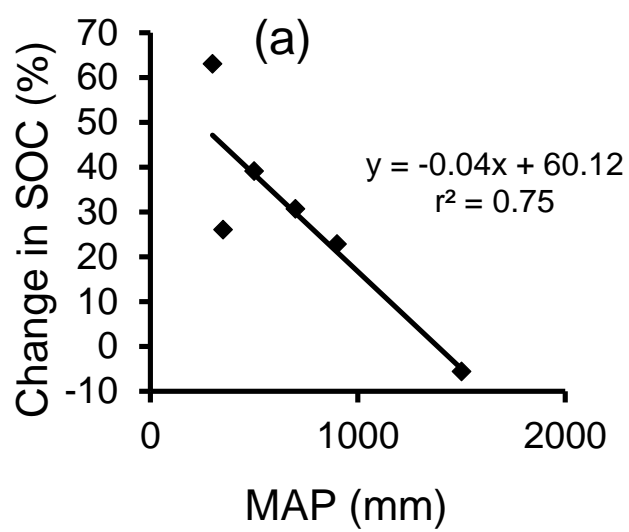


Figure 1



5 Figure 2

6

1           **CHAPTER 5: SOIL MICROBIAL BIOMASS AND FUNCTIONAL**  
2           **DIVERSITY IN SHRUB-ENCROACHED GRASSLANDS ALONG A**  
3           **PRECIPITATION GRADIENT**

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10  
11   This chapter has been formatted for *Soil Biology and Biogeochemistry*. A.M. conceived the  
12   study presented, collected, analysed the data and wrote the manuscript. D.W. contributed  
13   substantially to experimental design, data analysis and manuscript preparation.

## Abstract

The use of soil microbial and biochemical parameters could play an important role in monitoring effects of woody plant encroachment in grasslands due to their rapid reaction to environmental fluctuations. We compared total organic carbon ( $C_{org}$ ), soil total nitrogen (TON), microbial biomass, basal respiration, soil microbial quotient ( $C_{mic}/C_{org}$ ) species and functional diversity between pairs of neighbouring, leguminous woody encroached and open grassland plots along a rainfall gradient (300 mm to 1500 mm mean annual precipitation (MAP)) in South Africa. Soil  $C_{org}$  increased in semi-arid regions while in humid regions (1500 mm MAP) it decreased due to shrub encroachment. Soil total nitrogen was generally higher in the shrub-encroached grasslands compared to open grasslands. Soil microbial biomass and basal respiration increased across all sites due to shrub encroachment. There was also a difference in microbial functional diversity between the encroached and adjacent open grasslands, which was most evident in the semi-arid regions. Our results suggest that land-cover change influences microbial properties along the rainfall gradient.

## Keywords

Grasslands, shrub encroachment, basal respiration, microbial biomass, species diversity, microbial functional diversity, precipitation gradient



## 1    **Introduction**

2  
3    Soils are a major terrestrial carbon sink, containing approximately three times more carbon  
4    (1 555 Pg C ) than in vegetation (650 Pg C) and twice as much that in the atmosphere  
5    (750 Pg C) (Batjes and Sombroek 1997, Jobbágy and Jackson 2000, Cook et al. 2014). Land  
6    cover has a marked effect on soil carbon as a result of the interactions between organic inputs  
7    and subsequent input mediated by soil microbes. Change in land cover can affect the amount  
8    of carbon stored in the soil. Furthermore, the direction of change is dependent on land-use  
9    history, climate, soil and vegetation type (Zeng et al. 2009).

10            Grasslands and savanna ecosystems are experiencing intensive land-cover change due  
11    to shrub encroachment. For example, Li et al. (2004) as cited by Zeng et al. (2009), reported  
12    that woodlands increased by  $1.9 \times 10^5$  ha while open grasslands and savannas decreased by  
13     $1.1 \times 10^5$  and  $6.6 \times 10^4$ , respectively, in Kezuo Zhong Banner, central China. Roques et al.  
14    (2001) reported a shrub-cover increase of 31 % in northern Swaziland. Such land-cover  
15    changes are of concern because of the strong influence of different land types on soil quality,  
16    natural resource supply and carbon sequestration (Zeng et al. 2009, Barger et al. 2011).

17            Most studies on shrub encroachment have focused on soil chemical properties while  
18    less attention has been paid to microbial activities (Ross et al. 1999, Chen et al. 2000, Zeng et  
19    al. 2009). The use of soil microbial parameters could play an important role in monitoring  
20    effects of woody plant encroachment in grasslands relative to soil physical and chemical  
21    properties due to the rapid reactions of the former to any environmental fluctuations (Gryta et  
22    al. 2014). Some of the microbial parameters that have been used to assess soil quality include  
23    soil microbial biomass (Araújo et al. 2008, Truu et al. 2008, Zeng et al. 2009), soil respiration  
24    (Zeng et al. 2014) and functional diversity of soil microbial community (Zak et al. 1994,  
25    Gryta et al. 2014). Soil microbial biomass, the living part of organic matter, comprises 1 % to

3 % of the total organic C in the soil (Truu et al. 2008), and is both a source of labile nutrients and an agent for the transformation of soil nutrients (Alvarez et al. 1998, Araújo et al. 2008). The dynamics of soil microbial biomass can be used to predict long-term trends of soil organic matter (Truu et al. 2008). Soil respiration is a measure of carbon dioxide (CO<sub>2</sub>) released from the soil from decomposition of soil organic matter (SOM) by soil microbes and respiration from plant roots and soil fauna. Soil respiration is an important indicator of soil health because it indicates the level of microbial activity, SOM content and its decomposition. Functional diversity of microbes can be examined from a variety of perspectives, paralleling concepts analogous to those of taxonomic diversity. The simplest aspect is substrate richness, i.e. the number of different substrates that are used by a microbial community (Zak et al. 1994).

One of the most effective methods of measuring the utilisation of different carbon substrates by microorganisms is the Biolog Ecoplates technique (Garland and Mills 1991, Zak et al. 1994). The protocol is based on patterns of community-level carbon-source utilization (catabolic diversity) that generate community-level physiological profiling data for comparison of environmental samples (Zak et al 1994). Ros et al. (2008) reported that, by determining the diversity of microbial heterotrophic functions related to C utilization, more relevant information on the roles of microorganisms in the ecosystem can be obtained. This method has proved useful as a highly reproducible means of studying soil microbial functional diversity and of differentiating between microbial communities in natural soils, such as those under different land uses (Ros et al. 2008).

In this study we predicted that: (1) Soil organic carbon (SOC) and soil total nitrogen would increase under woody plant stands relative to grassland (Archer et al. 2004, Blaser et al. 2014); (2) This increase in C and N content would result in an increase in soil microbial biomass and respiration (Liao and Boutton 2008); (3) The microbial quotient will decrease

1 with woody-plant encroachment due to the more recalcitrant nature of lignified woody litter  
2 compared to herbaceous litter (Liao and Boutton 2008); and (4) There is no similarity in  
3 bacterial functional diversity between encroached and open grasslands because of the  
4 difference in the carbon compounds produced by woody plants and herbaceous plants (Parfitt  
5 et al. 2003, Liao and Boutton 2008, Zeng et al. 2009).

## 6 **Methods**

### 7 **Study sites**

8  
9 The study was carried out in six sites across a rainfall gradient in South Africa. The sites  
10 were: KwaMbonambi (1500 mm MAP), Stanger (900 mm MAP), Bergville (800 mm MAP),  
11 Bloemfontein (500 mm MAP), Pniel (350 mm MAP) and Middelburg (300 mm MAP).  
12 Further details of the sites are listed in Table 1. The soil samples were collected in September  
13 2014 and February 2015.

### 14 **Soil sampling**

15  
16 Soil samples were collected in September 2014 (dry season) and in March 2015 (rainy  
17 season). At each site, 12 10 m x 10 m plots (six in grass-dominated and six in shrub-  
18 encroached plots) were selected. The plots at each site had the same soil type and were on a  
19 level topography. In each plot, after removing the plant litter, five soil samples were  
20 randomly collected from 0-10 cm depth. The soil samples in each plot were then bulked and  
21 divided into two subsamples. One set of subsamples was used to determine SOC and TN and  
22 the other set was used to determine soil microbial properties. Samples designated for  
23 microbial properties were placed in an icebox until arrival at the laboratory and stored at 4 °C  
24 until analysis. The soil samples were transported to the laboratory within 24 h and analysis of

microbial properties began 24 h after arrival. The soil cores designated for SOC and TN were air-dried and passed through a 2 mm sieve. To remove soil inorganic C, soil samples were treated with 1N H<sub>2</sub>SO<sub>4</sub>/5% FeSO<sub>4</sub> and double-checked for complete inorganic C removal. SOC was measured using a Europar elemental analyser at BemLabs (Somerset West, Western Cape). For soil respiration, the Solvita® gel system measures the CO<sub>2</sub> burst that results from controlled drying-rewetting of soil (Haney et al. 2008). The Solvita gel system involves weighing 40 g of wetted soil samples and placing the weighed sample in 8 oz glass jars with a Solvita gel paddle. At the end of 24 h each paddle was placed in a digital colour reader (DCR) for spectrophotometric analysis.

Soil microbial biomass C (C<sub>mic</sub>) was determined by the chloroform fumigation–extraction method (Vance et al. 1987). Three replicate 25-g (fresh weight) portions of soils were weighed into 100 mL beakers and fumigated with ethanol-free chloroform under dark for 24 h at 25 °C. After the fumigant was removed, the soil was extracted with 100 mL 0.5 mol L<sup>-1</sup> K<sub>2</sub>SO<sub>4</sub> for 30 min. Similarly, the unfumigated soil was extracted to determine the background soluble C level. The organic C concentration in the soil extracts was measured by dichromate oxidation, and C<sub>mic</sub> was calculated as:  $C_{mic} = EC / kEC$ , where EC is the difference in soluble organic C concentration between the fumigated soil and unfumigated soil, and the kEC used was 0.38 to account for the extraction efficiency (Vance et al. 1987). The microbial quotient (C<sub>mic</sub> / C<sub>org</sub>) was obtained by dividing the mean microbial biomass by the corresponding organic carbon concentration.

For the determination of functional diversity, Biolog Ecoplates (Biolog, Hayward, CA, U.S.A.) were used. Biolog Ecoplates plates contain three replications of the 31 substrates known to commonly occur in plant root exudates and a control well across each plate. The number and diversity of substrates in these plates is sufficient to distinguish between different microbial communities in environmental samples (Ros et al. 2008). A method adapted from

Govaerts et al. (2007) was used to determine the community-level physiological profiling of the bacterial communities in the soil samples. A short incubation period at high inoculum density was chosen to reduce both the enrichment effect (Garland 1996) and the risk of contamination (Govaerts et al. 2007). Within 24 h of sample collection, 10 g of field-moist soil from each soil sample was added separately to 500 ml Erlenmeyer flasks containing 90 ml of a sterile saline solution (0.85 % w/v NaCl). The resultant soil suspensions were shaken at 150 rpm for 1 h at room temperature, and thereafter allowed to settle for 30 min.

When using the Biolog Ecoplates to determine microbial catabolism of carbon substrates, optical density is measured (change in well colour) over time. However, the presence of soil particles in the bacterial suspensions is problematic because it increases optical density. Therefore, to reduce and standardize the initial inoculum OD<sub>590</sub>, a serial dilution of the soil suspensions to  $1 \times 10^{-3}$  microbial colonies was carried out (Calbrix et al. 2005, Ros et al. 2008). Each soil suspension was first diluted to  $10^{-2}$  in 9 ml aliquots of sterile saline and finally to  $10^{-3}$  by adding 1 ml of the  $10^{-2}$  dilution to 90 ml sterile saline. Immediately prior to Ecoplate inoculation, the  $10^{-3}$  dilutions were thoroughly mixed and poured into separate, sterile, glass petri dishes. Aliquots (150 µl) of the soil suspensions were simultaneously inoculated into each Ecoplate, using a pipette. The rate of substrate utilization is indicated by the reduction of tetrazolium, which changes from colourless to purple. A ChroMate Model 4300 microplate reader was set at OD<sub>590</sub> nm, which is the peak absorbance for tetrazolium dye. Readings were taken over 5 days at 24, 48, 72, 96 and 120 h. Between readings, the microplates were incubated in the dark at 25 °C. A single time point (in this study, 120 h) was selected for statistical analysis. This was determined by the rate of colour development in the wells and the time by which most of the substrates were used (Garland 1996).

## Data analysis

Raw difference data for each Biolog Ecoplate were obtained by subtracting the colour response of the control wells (without a C source) from that of each of the substrate containing wells. Values were calculated separately for each plate at each time point (Govaerts et al. 2007). Wells showing very little colour response sometimes gave negative absorbance values after normalisation. Such negative numbers were set to zero, as recommended by several authors (Liu et al. 2007, Weber et al. 2007). Prior to statistical analysis, to eliminate variation in well colour-development caused by different cell densities, the average well colour-development (AWCD) value for each sample at each time point, was calculated according to Garland and Mills (1991), as follows:

$$AWCD = \Sigma (C - R) / n \quad (1)$$

where  $C$  is the colour production within each well (optical density measurement),  $R$  is the absorbance value of the plate's control well and  $n$  is the number of substrates (Biolog Ecoplates,  $n = 31$ ) (Choi and Dobbs 1999, Liu et al. 2007).

We also calculated the Shannon diversity index ( $H$ ) (Zak et al. 1994, Zhao et al. 2013) after 120 h of incubation.  $H$  was calculated as follows:

$$H = \sum p_i * \ln p_i \quad (2)$$

where  $p_i = \frac{n_i}{N}$ ,  $n_i$  is the AWCD of a particular substrate and,  $N$  is the sum of the AWCD of all substrates at 120 h and  $\ln$  is the natural logarithm.

As commonly used for Biolog Ecoplate data analysis, principal component analysis (PCA) was used to extract the main factors influenced by woody plant encroachment (Zak et al. 1994, Zhao et al. 2013). Prior to PCA, the data were transformed by dividing, for each

substrate, the difference in optical density at 120 h relative to the control wells, by the AWCD of the plate at 120 h, that is,

$$(C - R) / AWCD \quad (3)$$

where C, R and AWCD are as defined in equation (1). This further reduced the bias between samples caused by different initial inoculum densities, and resulted in standardized patterns being compared rather than absolute values (Garland and Mills 1991). An OD<sub>590</sub> threshold value of 0.1 (significant catabolic activity) was chosen for analysis, because lower values could not reliably be ascribed to bacterial activity but rather to 'noise' (i.e., no catabolic activity) (Heuer and Smalla 1997).

## Results

Soil organic carbon in the most humid sites (1500 mm MAP) were significantly higher ( $F_{1,6} = 211.50$ ,  $P < 0.001$ ) in the open grasslands than in encroached sites. At the more arid sites (350 and 300 mm MAP sites of Pniel and Middelburg, respectively), the shrub-encroached grasslands were significantly higher ( $F_{1,6} = 33.73$ ,  $P = 0.001$  and  $F_{1,6} = 8.19$ ,  $P = 0.029$ ) in soil organic carbon than in open grasslands (Figure 1).

At the most humid site (1500 mm MAP) total soil nitrogen was significantly higher in the shrub-encroached grassland than in the open grassland. Total soil nitrogen was also significantly higher at the 500 mm MAP site ( $F_{1,6} = 6.07$ ,  $P = 0.049$ ), the 350 mm site ( $F_{1,6} = 6.08$ ,  $P = 0.038$ ) and at the 300 mm site ( $F_{1,6} = 4.53$ ,  $P = 0.042$ ) (Figure 2).

Respiration before and during the rainy season was generally higher in the shrub-encroached sites than in the adjacent open grasslands at the 800, 500, 350 and 300 mm MAP sites (Table 2). Microbial biomass had a similar trend with soil respiration: there was generally an increase in microbial biomass due to shrub encroachment during the rainy

season in the 800, 500, 350 and 300 mm MAP sites (Table 2). Microbial quotient ( $C_{mic}/C_{org}$ ) was significantly lower in the shrub-encroached grasslands compared to open grasslands in semi-arid grasslands (500, 350 and 300 mm MAP).

The average well-colour development (AWCD) of the Ecoplates changes over time are shown in Table 3. Soil microbial activity increased with incubation time during the 120 h period. After 24 h, the AWCD was significantly higher in the shrub-encroached sites at 900 and 800 mm MAP ( $F_{1,10} = 227.21$ ,  $P < 0.00$  and  $F_{1,10} = 6.84$ ,  $P = 0.026$ , respectively). After 48 h, the shrub-encroached ecosystems at most sites had a higher AWCD than their paired adjacent open grasslands. After 72 h, only shrub-encroached sites at 500, 350 and 300 mm MAP (= drier sites) showed significantly different AWCD than adjacent open grasslands (Table 3). After 120 h, only shrub-encroached sites in the two driest sites, 350 mm and 300 mm MAP, had a significantly higher AWCD ( $F_{1,10} = 62.97$   $P < 0.001$  and  $F_{1,10} = 16.37$ ,  $P = 0.002$ , respectively) than in open grasslands.

At the 350 mm MAP site, the Shannon diversity index calculated after 120 h of incubation was significantly higher ( $F_{1,10} = 108.26$   $P < 0.001$ ) in the shrub-encroached areas ( $4.80 \pm 0.08$ ) than in adjacent grasslands ( $4.40 \pm 0.08$ ). The Shannon diversity index calculated after 120 h of incubation at the 300 mm MAP site was also significantly higher ( $F_{1,10} = 10.05$ ,  $P = 0.01$ ) in the encroached areas ( $4.81 \pm 0.08$ ) than in adjacent open grasslands ( $4.45 \pm 0.08$ ).

We used PCA plots to explain mean well-colour development. PC1 accounted for 23.5, 62.0, 34.9, 39.5, 34.3 and 38.6 % of the variance for the 1500, 900, 800, 500, 350 and 300 mm MAP, respectively (Figure 3). PC2 explained 21.3, 12.5, 24.2, 19.7, 24.6 and 14.2 % of the variance for those same plots. There was high variation in the open grasslands across all sites except in 1500 mm MAP site where the shrub-encroached ecosystem showed higher variation. There was some overlap between encroached and open grasslands at 1500, 900,



800 and 500 mm MAP (Figure 3a to 3d) which may indicate some degree of similarity in the species composition between the two land-cover systems. In the driest sites (350 and 300 mm MAP), the microbial communities differed between the encroached and unencroached grasslands (Figure 3e and 3f).

## Discussion

The findings of this study showed that there was loss of carbon due to shrub encroachment in KwaMbonambi while in semi-arid regions (Middelburg and Pniel) there was an increase in soil carbon with encroachment. These findings are similar to the study done in the same sites, which found that humid sites lost carbon while arid sites gained carbon due to shrub encroachment (Mureva et al., Unpublished). The loss in carbon at KwaMbonambi could be due to the areas under grassland being highly productive and allocating a large proportion of SOC belowground compared to encroached lands (Jackson et al. 2002). The increase of SOC in the semi-arid sites (350 and 300 mm MAP) is in agreement with the well-described island of fertility (Liao et al. 2006), whereby shrubs and trees develop extensive root systems that track nutrients from surroundings, leading to local nutrient recycling and organic-matter accumulation (Belsky et al. 1989). The accumulation of SOC in the shrub-encroached grassland in the semi-arid sites can also be due to higher rates of primary productivity in the soil beneath deeply rooted shrubs that increase soil organic matter input through surface litter and accumulation of complex organic compounds, which are resistant to decay (Liao et al. 2006).

The higher soil total nitrogen (STN) stocks in encroached sites than in open grasslands is in agreement with a number of studies from other African savanna ecosystems,

1 where shrub-encroached sites have been compared with open grasslands (Ludwig et al. 2004,  
2 Hagos and Smit 2005, Blaser et al. 2014). The increase in TN can be attributed to the  
3 presence of nitrogen-fixing species (Bush and Van Auken 1986, Pugnaire et al. 1996, Stock  
4 et al. 2005), higher net-primary productivity and more biomass in the encroached areas than  
5 in open grasslands (Boutton and Liao 2010).

6 Our prediction that there is increased soil microbial respiration and biomass was  
7 supported by our findings. This study showed that soil microbial respiration and microbial  
8 biomass generally increased due to woody plant encroachment across all sites during the  
9 rainy season. Our findings are also in agreement with other studies on soil respiration in  
10 encroached grasslands (McCulley et al. 2004, Liao and Boutton 2008). Differences in soil  
11 respiration and microbial biomass between the two land-cover types (i.e. encroached and  
12 open grasslands) indicate that soil C availability was altered by land-cover change at these  
13 sites. As soil moisture and temperature were controlled during the laboratory incubation, the  
14 difference in respiration rates among the two land-cover types would have occurred due to  
15 changes in factors such as substrate quality and availability (Parfitt et al. 2003, Zeng et al.  
16 2009). C respiration is mostly controlled by the availability of easily decomposable soil  
17 organic C (Menyailo et al. 2002). Increases in soil-resource availability are commonly  
18 observed with shrub encroachment in arid and semi-arid regions (Jackson et al. 2002, Billings  
19 2006, Liao et al. 2006, Liao and Boutton 2008). Differences in microbial biomass in the  
20 semi-arid regions might have been due to the higher input of soil organic carbon in the  
21 encroached ecosystems than in the unencroached ecosystems (Chen et al. 2000, Vance and  
22 Chapin 2001). Our findings in two intermediate precipitation sites of Bergville and  
23 Bloemfontein showed an increase in microbial biomass in the shrub-encroached areas even  
24 though there was no significant difference in soil organic carbon. We also found that there  
25 was higher coarse particulate organic matter (cPOM) in the encroached grasslands compared

1 to the adjacent open grasslands (Mureva et al. Unpublished). cPOM is a highly labile soil  
2 carbon fraction and is thought to represent partly decomposed plant material at an early stage  
3 of decomposition (Leifeld and Kögel-Knabner 2005). Increase in this fraction increases  
4 substrate quantity and availability which increases soil respiration (Wang et al. 2003) and  
5 microbial biomass in turn (Chen et al. 2000).

6 We predicted that the  $C_{mic}/C_{org}$  ratio would be lower in encroached than in open  
7 grasslands due to the inherently more recalcitrant nature of woody litter compared with  
8 herbaceous litter. In the semi-arid regions, the lower  $C_{mic}/C_{org}$  ratios in encroached compared  
9 to open grasslands (Table 2) supported our hypothesis and suggested a decrease in the  
10 relative availability of organic matter inputs with encroachment (i.e. less microbial biomass is  
11 supported per unit SOC).

12 Although organic matter input may be significantly higher in the encroached  
13 grassland, quality of the C may be poorer due to the higher concentration of lignin and plant  
14 secondary compounds than in the open grasslands (Liao and Boutton 2008). A study on  
15 below-ground carbon storage showed that litter and roots of woody species contain higher  
16 levels of aliphatic biopolymers (primarily cutin and suberin) than those in open grasslands  
17 (Boutton et al. 2009). These aliphatic biopolymers comprise a principally important subset of  
18 recalcitrant material in soils (Nierop and Verstraten 2003, Mikutta et al. 2006). Their greater  
19 abundance provides evidence that organic matter inputs in wooded areas are biochemically  
20 more resistant to decomposition than those in grassland (Liao and Boutton 2008). Another  
21 study on soil respiration indicated longer mean residence times for SOC in encroached areas  
22 than in open grasslands (Boutton et al. 2002, McCulley et al. 2004), suggesting that a larger  
23 proportion of woodland-derived C inputs is resistant to decomposition than in open  
24 grasslands. Collectively, these prior studies support the notion that lower  $C_{mic}/C_{org}$  ratios in

wooded areas are due to poorer quality organic matter inputs and stores that are less accessible to serve as substrate for the production of microbial biomass.

The average well-colour development (AWCD), which reflected the carbon utilisation of a soil microbial community (Zak et al. 1994, Ros et al. 2008) showed differences in microbial activity in semi-arid regions after 120 h of incubation. The AWCD at 120 h was higher in the shrub-encroached ecosystems than in the adjacent open grassland in the driest sites (Middelburg and Pniel). The higher AWCD at the encroached sites implies that soil microbial metabolism in the encroached sites greater and the activity intensity increased more than in the open grasslands. This would lead to rapid consumption of carbon source by shrub-encroached microbes to maintain their physiological needs (Yang et al. 2006).

The PCA indicated a difference between functional diversity of the encroached and unencroached ecosystems in the two driest sites (Middelburg and Pniel). This shows differences in functional diversity in the two communities. The difference in functional diversity could be due to the difference in quantity and quality of substrate inputs into the soil and amended environmental conditions under shrubs (Liao and Boutton 2008). Under tree and shrub canopies, there is reduced solar irradiance by 45-65 %, soil temperatures by 5-11 °C, and rainfall by 0-50% compared to open grasslands (Belsky et al. 1989).

## **Conclusions**

Soil microbial biomass and soil respiration increased with woody plant encroachment, probably due to the greater above and below ground productivity relative to open grasslands, resulting in greater organic inputs. The high microbial biomass and respiration in the encroached grasslands indicate that there is more carbon loss than in the open grasslands. However, the accumulation of SOC and total N in woody communities implies that woody

1 plants have augmented inputs of organic matter to a greater extent than they have induced  
2 losses of C and N from these pools. The increase in SOC and STN may be a function of both  
3 greater input of poor quality organic matter that is relatively resistant to decay in woody  
4 communities than in open grasslands.

5

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**Table 1**

Study site descriptions.

Site	GPS coordinates	Annual Rainfall (mm)	Temperature (°C) Min Max		Biome	Soil Characteristics	Major plant species
KwaMbonambi	28° 49' 60.61"S 32° 16' 96.92"E	1500	3.5	35	Maputaland wooded grasslands	Quaternary redistributed sands supporting yellowish redistributed sands of the Berea formation	<i>Sporobolus fimbriatus</i> , <i>Digitaria natalensis</i> (grasses); <i>Diospyros lycioides</i> (shrub); <i>Terminalia sericea</i> (tree)
Stanger	29° 18' 59.05" S 31° 22' 13.24"E	900	5.8	32.6	KwaZulu- Natal Coastal Belt	Ordovician Natal group sandstone	<i>Themeda triandra</i> , <i>Aristida junciformis</i> (grass); <i>Acacia karroo</i> (tree)
Bergville	28° 79' 06.30" S 29° 38' 98.40"E	700	5.8	32.6	KwaZulu- Natal moist grasslands	Ordovician Natal group sandstone	<i>Themeda triandra</i> , <i>Hyparrhenia hirta</i> (grasses); <i>Acacia karroo</i> , <i>A sieberiana</i> (trees)
Bloemfontein	28° 59' 16.77"S 26° 16' 54.24E	450	0	32	Bloemfontein dry grasslands	Sedimentary mudstones and layers of sandstone	<i>Aristida congesta</i> , <i>A. diffusa</i> , <i>Cynodon dactylon</i> (grass); <i>Acacia karroo</i> (tree)
Pniel	28° 34' 50.00"S 24° 30' 30.70"E	350	-4.1	37.5	Kimberley thornveld	Sandy to loam soils of the Hutton soil form	<i>Eragrotis curvula</i> , <i>Schmidtia pappophoroides</i> (grass); <i>Acacia erioloba</i> , <i>A tortilis</i> , <i>A. karroo</i> , <i>A. mellifera</i> , <i>Tarchonanthus camphoratus</i> (trees)
Middelburg	31° 25' 98.83"S 24° 58' 82.10"E	300	-7.2	36.1	Eastern Upper Karoo	Sandy to loam soils of the Hutton soil form	<i>Aristida</i> , <i>Eragrostis</i> (grasses); <i>Searsia erosa</i> , <i>S. burchellii</i> , <i>Diospyros lycioides</i> , <i>Eriocephalus ericoides</i> (shrubs)

**Table 2**

Soil respiration, microbial biomass and soil microbial quotient ( $C_{mic}/C_{org}$  %) in open (unencroached) grassland (Grass) and shrub-encroached grasslands (Shrub) across six grassland sites in September 2014 and February 2015. Numbers in bold indicate significant differences at  $P = 0.05$  between encroached (shrub) and open grasslands (grass) within the same period. S.E = standard errors.

Site	Land cover	mean $\pm$ S.E Respiration (ppm)		mean $\pm$ S.E Microbial biomass (mg/kg)		mean $\pm$ S.E $C_{mic}/C_{org}$ (%)	
		Feb	Sept	Feb	Sept	Feb	Sept
<b>KwaMbonambi</b> (1500 mm MAP)	Shrub	<b>5.5 <math>\pm</math> 0.69</b>	2.5 $\pm$ 0.41	<b>118.3 <math>\pm</math> 20.4</b>	83.33 $\pm$ 8.2	0.8 $\pm$ 0.25	0.7 $\pm$ 0.09
	Grass	<b>4.3 <math>\pm</math> 0.54</b>	2.8 $\pm$ 0.36	<b>120 <math>\pm</math> 10.95</b>	86.67 $\pm$ 10.32	0.8 $\pm$ 0.11	0.6 $\pm$ 0.038
<b>Stanger</b> (900 mm MAP)	Shrub	16.1 $\pm$ 1.77	21.1 $\pm$ 2.05	383.2 $\pm$ 36.11	493.3 $\pm$ 45.2	2.4 $\pm$ 0.27	3.04 $\pm$ 0.33
	Grass	18.1 $\pm$ 1.87	20.7 $\pm$ 2.2	428.3 $\pm$ 64.6	470 $\pm$ 102.57	2.6 $\pm$ 0.43	2.8 $\pm$ 0.60
<b>Bergville</b> (700 mm MAP)	Shrub	<b>21.7 <math>\pm</math> 1.89</b>	<b>33.6 <math>\pm</math> 3.56</b>	<b>506.7 <math>\pm</math> 43.7</b>	<b>766.7 <math>\pm</math> 77.9</b>	1.15 $\pm$ 0.2	1.74 $\pm$ 0.91
	Grass	<b>17.9 <math>\pm</math> 2.07</b>	<b>21.4 <math>\pm</math> 1.46</b>	<b>420 <math>\pm</math> 44.27</b>	<b>496.7 <math>\pm</math> 30.11</b>	1.0 $\pm$ 0.19	1.24 $\pm$ 0.23
<b>Bloemfontein</b> (500 mm MAP)	Shrub	<b>34.4 <math>\pm</math> 3.94</b>	<b>23.5 <math>\pm</math> 2.49</b>	<b>786.7 <math>\pm</math> 85.24</b>	<b>546 <math>\pm</math> 55.02</b>	<b>8.8 <math>\pm</math> 1.32</b>	<b>6.13 <math>\pm</math> 0.71</b>
	Grass	<b>10.3 <math>\pm</math> 1.68</b>	<b>14.5 <math>\pm</math> 1.92</b>	<b>251.67 <math>\pm</math> 36.0</b>	<b>345.0 <math>\pm</math> 41.35</b>	<b>3.5 <math>\pm</math> 0.67</b>	<b>4.73 <math>\pm</math> 0.65</b>
<b>Pniel</b> (350 mm MAP)	Shrub	<b>3.53 <math>\pm</math> 0.23</b>	<b>4.8 <math>\pm</math> 0.30</b>	<b>103.3 <math>\pm</math> 12.11</b>	<b>133.3 <math>\pm</math> 5.16</b>	<b>4.9 <math>\pm</math> 0.12</b>	<b>6.44 <math>\pm</math> 0.04</b>
	Grass	<b>2.4 <math>\pm</math> 0.33</b>	<b>3.48 <math>\pm</math> 0.26</b>	<b>83.33 <math>\pm</math> 8.17</b>	<b>105.0 <math>\pm</math> 8.37</b>	<b>5.3 <math>\pm</math> 0.17</b>	<b>6.7 <math>\pm</math> 0.07</b>
<b>Middelburg</b> (300 mm MAP)	Shrub	<b>4.6 <math>\pm</math> 0.44</b>	3.3 $\pm$ 0.44	<b>126.3 <math>\pm</math> 7.14</b>	98.33 $\pm$ 2.53	<b>3.06 <math>\pm</math> 1.0</b>	<b>2.37 <math>\pm</math> 0.32</b>
	Grass	<b>2.9 <math>\pm</math> 0.2</b>	3.1 $\pm$ 0.38	<b>94.2 <math>\pm</math> 6.65</b>	98.30 $\pm$ 9.83	<b>3.6 <math>\pm</math> 0.34</b>	<b>3.73 <math>\pm</math> 0.44</b>

**Table 3**

Average well colour-development (mean AWCD  $\pm$  standard error) of soil microbial communities for different incubation times. Numbers in bold indicate significant differences at  $P = 0.05$  between encroached (shrub) and open grasslands (grass) within the same period.

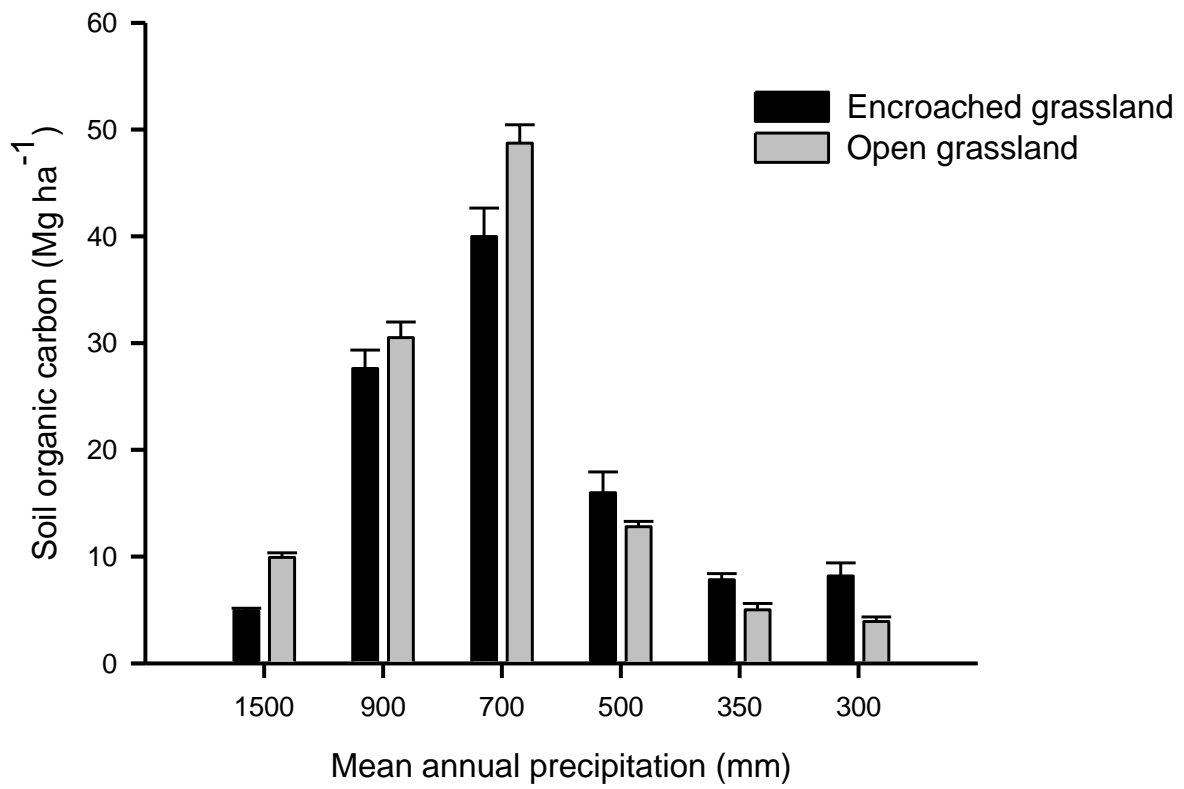
Site	Land cover	Time (Hours)				
		24	48	72	96	120
<b>KwaMbonambi</b> (1500 mm MAP)	Grass	0.08 $\pm$ 0.01	<b>0.39<math>\pm</math>0.03</b>	0.74 $\pm$ 0.03	<b>0.90<math>\pm</math>0.07</b>	0.96 $\pm$ 0.08
	Shrub	0.08 $\pm$ 0.02	<b>0.49<math>\pm</math>0.09</b>	0.75 $\pm$ 0.05	<b>0.74<math>\pm</math>0.08</b>	0.89 $\pm$ 0.05
<b>Stanger</b> (900 mm MAP)	Grass	<b>0.01<math>\pm</math>0.00</b>	<b>0.59<math>\pm</math>0.03</b>	0.82 $\pm$ 0.10	0.98 $\pm$ 0.09	1.14 $\pm$ 0.07
	Shrub	<b>0.05<math>\pm</math>0.01</b>	<b>0.69<math>\pm</math>0.05</b>	0.91 $\pm$ 0.11	1.04 $\pm$ 0.11	1.18 $\pm$ 0.09
<b>Bergville</b> 700 mm MAP	Grass	0.02 $\pm$ 0.00	0.45 $\pm$ 0.07	0.80 $\pm$ 0.16	0.90 $\pm$ 0.08	1.01 $\pm$ 0.06
	Shrub	0.02 $\pm$ 0.00	0.48 $\pm$ 0.05	0.78 $\pm$ 0.13	0.90 $\pm$ 0.20	1.06 $\pm$ 0.08
<b>Bloemfontein</b> (500 mm MAP)	Grass	0.01 $\pm$ 0.00	<b>0.31<math>\pm</math>0.02</b>	<b>0.60<math>\pm</math>0.07</b>	0.67 $\pm$ 0.17	<b>0.81<math>\pm</math>0.01</b>
	Shrub	0.01 $\pm$ 0.00	<b>0.39<math>\pm</math>0.02</b>	<b>0.69<math>\pm</math>0.05</b>	0.84 $\pm$ 0.09	<b>0.96<math>\pm</math>0.10</b>
<b>Pniel</b> (350 mm MAP)	Grass	0.04 $\pm$ 0.00	<b>0.24<math>\pm</math>0.03</b>	<b>0.45<math>\pm</math>0.05</b>	<b>0.61<math>\pm</math>0.06</b>	<b>0.64<math>\pm</math>0.05</b>
	Shrub	0.04 $\pm$ 0.00	<b>0.51<math>\pm</math>0.06</b>	<b>0.66<math>\pm</math>0.07</b>	<b>0.82<math>\pm</math>0.10</b>	<b>0.91<math>\pm</math>0.07</b>
<b>Middelburg</b> (300 mm MAP)	Grass	0.05 $\pm$ 0.00	<b>0.28<math>\pm</math>0.02</b>	<b>0.52<math>\pm</math>0.03</b>	<b>0.64<math>\pm</math>0.04</b>	<b>0.65<math>\pm</math>0.07</b>
	Shrub	0.05 $\pm$ 0.01	<b>0.47<math>\pm</math>0.03</b>	<b>0.66<math>\pm</math>0.09</b>	<b>0.79<math>\pm</math>0.11</b>	<b>0.86<math>\pm</math>0.05</b>

**Figure captions**

**Fig 1.** Comparison of soil organic carbon at 0-10 cm depth between encroached site and adjacent grasslands along a precipitation gradient.

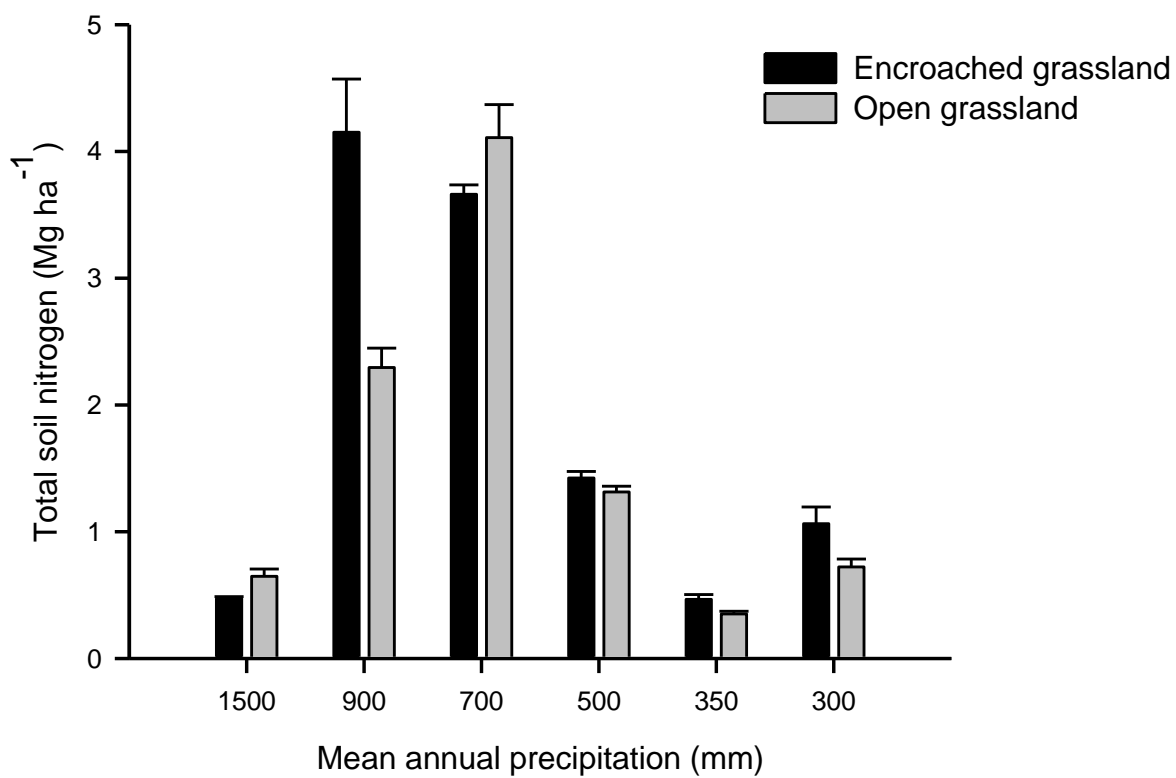
**Fig 2.** Comparison of total soil nitrogen at 0-10 cm depth between encroached site and adjacent open grasslands along a precipitation gradient.

**Fig 3.** PCA of the first two principal components axes (PC1 and PC2) of Biolog Ecoplate profiles from microbial communities at (a) KwaMbonambi (1500 mm MAP), (b) Stanger (900 mm MAP), (c) Bergville (700 mm MAP), (d) Bloemfontein (500 mm MAP), (e) Pniel (350 mm MAP), and (f) Middelburg (300 mm MAP) after 120 h of incubation. Dark triangles represents functional diversity in the encroached grasslands while circles represent functional diversity in the open grasslands. There were some differences between functional diversity of the encroached and open grassland in the two driest sites (Middelburg and Pniel).

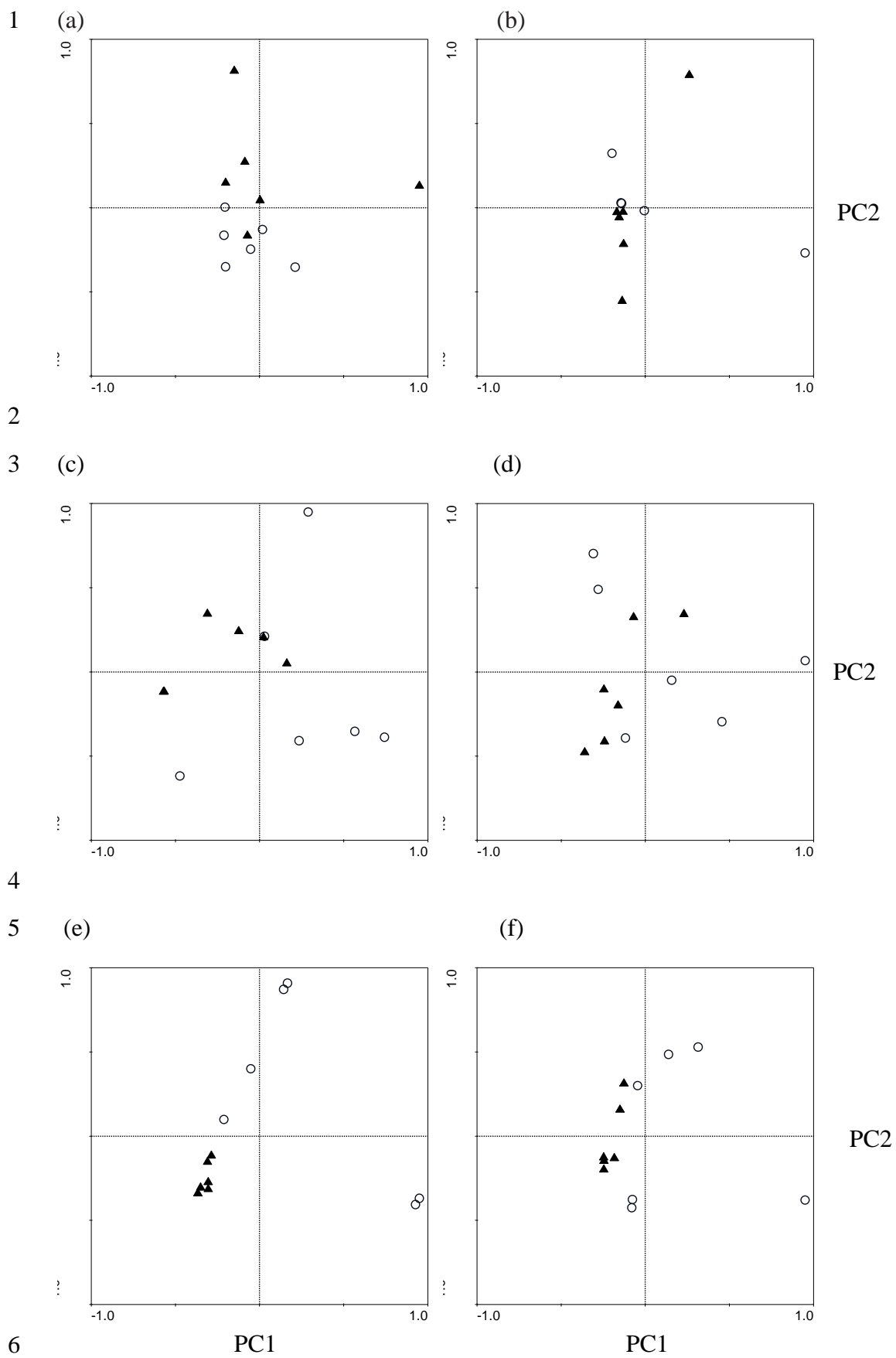


**Fig 1.**





**Fig 2.**



## CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER RESEARCH

### Introduction

The increase of woody plants often unpalatable to grazers (woody plant encroachment; also called shrub or bush encroachment) is a widespread economic and ecological problem in savannas (Bond 2008, Van Auken 2009, Ward et al. 2014). This is a problem affecting 10-20 million ha of South Africa (Hoffman and Ashwell 2001) but also affects savannas across the world (Archer et al. 1995, Bond 2008). Although heavy grazing, fire intensity and frequency, soil moisture, nutrients and global climate change have been attributed to grassland invasion by woody species (Ward 2005, Bond 2008, Van Auken 2009), the mechanisms behind woody plant encroachment are poorly understood (Ward 2005). This shift in vegetation structure has altered C and N cycling. However, whether the affected ecosystems are a net source or sink of atmospheric CO<sub>2</sub> remains controversial (Goodale and Davidson 2002). Given the ecological and economic importance of grasslands, more extensive and robust studies of mechanisms that drive the increase in woody density in grasslands and how this increase in woody species affects the C and N cycle are still warranted (Goodale and Davidson 2002, Ward 2005).

### General Conclusions from this Study

Woody plant density was significantly higher in continuous and summer grazing plots compared to winter grazing plots (**Chapter 2**). The higher densities in the wet-season grazing plots indicate that the removal of grass in the wet season causes a reduction in grass density, reducing competition and allowing the woody species to thrive. Although grazing in the dry season also reduces plant density and competition, there is limited moisture for woody species

1 to germinate and become established. There is therefore high shrub density in plots where  
2 grazing occurs during the wet season rather than in the dry season. The spatial pattern of the  
3 encroaching woody species was generally one of aggregation across all the grazing plots  
4 **(Chapter 2)**. This aggregation might be due to facilitation and/or spatial heterogeneity. The  
5 aggregated woody plants in semi-arid grasslands may create an ‘island of fertility’, enhancing  
6 the growth of other woody species (Ravi et al. 2010).

7 My study showed that wood plant encroachment into grasslands changes soil organic  
8 carbon and nitrogen storage **(Chapter 3 and Chapter 4)**. This study showed a negative  
9 correlation between changes in soil organic C in the 0-100 cm soil layer and mean annual  
10 precipitation (MAP). The most humid site (1500 mm MAP) had less C in shrub-encroached  
11 sites while the drier sites (300-350 mm MAP) had more C than their paired open grasslands.  
12 This finding implies that soils in shrub-encroached semi-arid regions sequester more C while  
13 humid sites sequester more C in open grasslands. I also found that, although C<sub>3</sub> plants  
14 dominate the encroached grasslands, the bulk of the SOC is still from the C<sub>4</sub> grasses  
15 **(Chapter 3)**. The increase in the C<sub>4</sub> contribution to SOC with depth may be an indication that  
16 these encroached sites were once open grasslands, as was shown independently for at least  
17 three of the study sites (1500 mm, 350 mm and 350 mm MAP sites).

18 The study also showed that soil physical fractions along a depth profile were better  
19 able to capture the effects of woody plant encroachment on C and N storage than bulk soil C  
20 and N samples, which showed no significant differences **(Chapter 4)**. In our mesic sites,  
21 SOC and N in bulk soil were generally not significantly different between encroached and  
22 open grasslands. However, in these mesic sites there were some differences when I compared  
23 SOC in the cPOM, Magg and silt and clay fractions.

I also found that the change in SOC due to shrub encroachment was mainly driven by changes in the coarse particulate organic matter (cPOM) fraction (**Chapter 4**). Change in SOC was lowest in the silt and clay fraction, implying that woody plant encroachment in our study sites may be fairly recent because it takes hundreds of years for SOC in the silt and clay fraction to change (Throop et al. 2013). This result is supported by Ward et al. (2014) who showed that shrub encroachment substantially increased from 1993 onwards. This also confirms my findings in **Chapter 2** where I used stable carbon isotopes that showed that the SOC in the encroached grasslands is mainly derived from C<sub>4</sub> plants.

Woody plant encroachment into grasslands increases soil respiration, microbial biomass and microbial species diversity (**Chapter 5**). The change in these microbial parameters is more pronounced in the semi-arid regions. Despite increases in soil respiration rates, microbial biomass and microbial species diversity, it appears that organic matter inputs have exceeded losses, such that these semi-arid areas are undergoing grassland-to-woodland succession and have been behaving as net C sinks.

## **Implications of the Study**

The implications of this study are: (1) the woody densities in the grazed wet-season plots increased even though the stocking density (2 sheep/ha) was within the recommended rates. This implies the stocking density of wet-season grazing plots should be reduced while the stocking rates of the dry-season grazing plots may be maintained at their current levels; (2) that future studies in soil C and N dynamics should also focus on C and N contained in different soil fractions; and (3) that this study enhances current understanding of the effects of woody plant encroachment on mechanisms of soil C and N sequestration in grasslands. Because similar grassland-to-woodland conversions are geographically extensive in grasslands and savannas worldwide, processes and mechanisms of soil C and N storage

documented here could have significance for understanding global C and N cycles and the earth's climate system.

### Future Research

I recognise that my findings on the mechanisms that drive woody plant encroachment (**Chapter 2**) are from an unreplicated grazing trial. I suggest that a greenhouse experiment be set up to validate these findings. I propose two greenhouse experiments. One of the experiments should test whether grazing at different intensities in the wet season causes shrub encroachment. The other experiment will test whether *S. erosa* has a nurse effect (i.e. facilitation) on *S. burchellii* and *D. lycioides* and whether this effect is due to shading, hydraulic lift and/or differential accumulation of nutrients or some combination of these factors.

I found that there was a general increase in soil total nitrogen with woody encroachment across sites (**Chapters 3, 4 and 5**). In most of the sites (1500, 900, 700, 500, and 350 mm MAP) this increase may be attributed to the presence of nitrogen-fixing plants. In the 300 mm MAP site, I found more nitrogen in the encroached grassland even though the encroaching woody species were non-nitrogen-fixers. It would be interesting to determine the mechanisms that woody plants that non-nitrogen-fixers employ that add nitrogen to the soil.

Using the mixed-isotope model, I proposed that the bulk of SOC in the encroached grasslands is from C<sub>4</sub> vegetation (**Chapter 3**). The mixed-isotope model does not take into consideration the change in the  $\delta^{13}\text{C}$  signature due to decomposition. Some studies have argued that decomposition does not affect  $\delta^{13}\text{C}$  signature (Cleveland et al. 2004, Boström et al. 2007) while others consider decomposition as important (Blair et al. 1985, Fernandez et.al. 2003). I therefore propose an experiment to determine the effects of decomposition on  $\delta^{13}\text{C}$

signature. This will help further clarify the role of shrub encroachment in carbon sequestration.

My study mainly focused on soil organic carbon and nitrogen changes due to woody plant encroachment (**Chapters 3 and 4**). However, above-ground and below-ground plant biomass carbon can be an important C sink (Baral et al. 2009, Basuki et al. 2009). The Kyoto Protocol (IPCC 2014) recognized the importance of woody plants in mitigating greenhouse gas emissions (i.e. carbon dioxide, methane and other compounds) and has included woody and soil C sequestration in the list of acceptable offsets (Baral et al. 2009). To correctly assess the potential of encroached grassland in carbon sequestration both below- and above-ground biomass need to be taken into consideration. I therefore recommend that changes in above- and below-ground biomass C due to woody plant encroachment in grasslands be determined.

Shrub encroachment in semi-arid regions altered the soil microbial functional diversity (**Chapter 5**). However, a limitation of natural experiments such as mine is that we cannot ascertain the proximate mechanisms driving changes in microbial functional diversity across habitats (Yannarell et al. 2014). Rather, we speculate that several dependent environmental drivers may operate in encroached-grassland systems. Some of these drivers are: (1) Differences in micro-climate between encroached and open grassland (Belsky et al. 1989, Smith and Johnson 2004); (2) Higher net-primary productivity in shrub-encroached than in open grasslands (García-Morote et al. 2012); and (3) Woody-plant root exudates and litter may provide novel substrates for soil microbes (Yannarell et al. 2014). Investigating different contributions of these various factors should be an active, albeit challenging area of soil microbial ecology research. Such investigations would allow us to know whether these changes in functional diversity are

1 brought about by direct plant-microbe interactions with shrubs (e.g., mycorrhizae) or  
2 through indirect channels mediated by litter or alterations to the soil environment  
3 (Yannarell et al. 2014).

4 Woody plant encroachment is viewed as having a negative impact on the economy  
5 (Bond 2008, Van Auken 2009, Ward et al. 2014). However, the existing economic valuations  
6 are heavily biased towards pastoral production systems and ignore emerging evidence of the  
7 importance of ecosystem services (such as carbon sequestration) that encroaching woody  
8 species provide, especially in semi-arid regions. I recommend that a proper economic  
9 valuation be done that takes both pastoral production systems and the ecosystem services  
10 provided by encroaching species into consideration. Such an evaluation will give a proper  
11 perspective of the economic challenges that woody plant encroachment is causing.

12



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