

Barriers to ecosystem restoration after clearing invasive *Acacia* species in the South African fynbos: soil legacy effects, secondary invaders and weedy native species

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Dissertation presented for the degree of Doctor of Philosophy in the Faculty of Science at
Stellenbosch University



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December 2018

Declaration

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Abstract

A significant proportion of the world's ecosystems are invaded by alien N₂-fixing woody species such as Australian acacias. Invasive alien N₂-fixing woody species often transform ecosystems through their negative impacts on soil chemistry, seed banks and microbial communities, and native plant diversity. Management interventions such as clearing are necessary to reduce these negative impacts. It is often assumed that clearing the invasive species will lead to a dissipation of their impacts and native plant diversity recovery. However, this is often not the case because the invasive species' negative impacts can become persistent soil legacy effects and present barriers to restoration of viable native plant communities. Understanding barriers to restoration can lead to improved restoration outcomes. Using *Acacia saligna* invasions in the South African fynbos as case study, this thesis explored soil legacy effects, secondary invasion and weedy native species dominance after clearing invasive acacias.

In chapter one, I reviewed global literature to understand how soil legacy effects of invasive alien N₂-fixing woody species present barriers to restoration, and identify management actions that could potentially be used to address them. In chapter two, I investigated how long soil legacy effects of invasive *A. saligna* persist after clearing using soil sample analyses. In chapter three, I explored the effect of invasive *A. saligna*'s soil chemical and biotic legacies, and weedy native species on native species re-establishment using a greenhouse experiment. In chapter four, I identified species that are secondary invaders after clearing invasive *A. saligna* across several sites and investigated the effects of vegetation type and fire application on their establishment over three years after clearing using vegetation monitoring. In chapter five, I investigated interactions between secondary invaders and the extent to which soil nitrate levels, apparent after clearing invasive *A. saligna*, influence secondary invasion and weedy native species dominance using growth chamber and greenhouse experiments.

I found that altered soil microbial communities, depleted native soil seed banks, elevated N status, secondary invasion and weedy native species dominance, and reinvasion can be barriers to restoration. Furthermore, management actions such as carbon addition, soil microbial treatments, herbicide or graminicide application and native species reintroduction can be used to address these barriers to restoration. *Acacia saligna*'s soil chemical legacies persisted up to ten years after clearing. However, they did not have direct negative

consequences on the re-establishment of native proteoid shrubs but soil biotic legacies and presence of weedy native species reduced their growth. Secondary invasion was not habitat specific, was promoted by fire application and persisted up to three years after clearing at levels similar to or higher than the first year. Lastly, growth of secondary invaders and weedy native species increased with an increase in soil nitrate levels.

I conclude that practicing restoration ecologists should manage soil legacy effects, secondary invaders and weedy native species after clearing invasive *A. saligna* to improve restoration outcomes.

Opsomming

'n Merkbare deel van die wêreld se ekosisteme word deur uitheemse houtagtige stikstofbindende plant spesies soos die Australiese Akasias ingedring. Hierdie indringers transformeer dikwels ekosisteme deur hul negatiewe impak op grondchemie, saadbanke, mikrobiële gemeenskappe en inheemse biodiversiteit. Om hierdie negatiewe impakte te bestry, is bestuurspraktyke vir die afkap van die uitheemse indringers nodig. Daar is 'n aanname dat die afkap van hierdie indringerspesies sal lei tot 'n einde aan hul impak en dat die inheemse biodiversiteit sal herstel. Dit is egter dikwels nie die geval nie, aangesien die negatiewe impak van die indringerspesies langdurige of permanente veranderinge in die grond kan nalaat en bied hindernisse vir die herstel en restorasie van inheemse plantgemeenskappe. Om hierdie veranderinge en hindernisse te verstaan, kan lei tot verbeterde veldrestorasie resultate. *Acacia saligna* indringing in die Suid-Afrikaanse fynbos word as gevallestudie gebruik in hierdie tesis om die effek te ondersoek van die veranderde nalating van N₂ in die grond, sekondêre indringers en die dominansie van onkruidagtige inheemse spesies.

In hoofstuk een het ek die globale literatuur nagegaan om te verstaan hoe grond-nalatakseffekte van stikstofbindende uitheemse indringerplante hindernisse tot veldherstel kan bied. Daar is ook bestuursaksies ge-identifiseer wat moontlik gebruik kan word om dit aan te spreek. In hoofstuk twee het ek deur ontleding van grondmonsters ondersoek hoe lank die stikstof grondnalatingseffekte van *A. saligna* voortduur na die skoonmaak van die indringers. In hoofstuk drie het ek met behulp van 'n kweekhuis-eksperiment, gekyk na die effek van *A. saligna* se nalatenskap op grondchemikalieë en grondbiotiese verwantskappe, en na die effek van onkruidagtige inheemse spesies op die hervestiging van inheemse spesies. In hoofstuk vier het ek sekondêre indringerspesies geïdentifiseer nadat *A. saligna* in verskeie areas skoongemaak is. Daar is na die effek van plantegroei tiepe, vuur-gebruik en die tydperk na die oorspronklike skoonmaak van indringers gekyk. Data is oor 'n drie-jaar tydperk ingesamel deur moniteringsplotte. In hoofstuk vyf het ek gebruik gemaak van groeikamer en kweekhuis eksperimente om ondersoek in te stel na interaksies tussen sekondêre indringerspesies. Ek het ook die mate waarin grondnitraatvlakke, wat na die verwydering van *A. saligna* voorkom, sekondêre indringing en onkruidagtige inheemse dominansie beïnvloed, ondersoek.

Veranderde grondmikrobiëse gemeenskappe, uitgeputte inheemse saadbank, verhoogde stikstof, sekondêre indringing en dominante onkruidagtige inheemsespesies, en her-investasie deur *A. saligna*, is potensiële hindernisse vir veldherstel. Bestuurstechnieke wat potensieel hierdie hindernisse kan oorbrug, sluit in koolstof byvoeging, grondmikrobiëse behandelings, onkruidodder- of grasdodertoediening en die hervestiging van inheemse spesies. *Acacia saligna* se grondnalatingseffekte bly teenwoordig tot tien jaar na die oorspronklike skoonmaak van die indringer. *Acacia saligna* se chemiese verandering van grond het nie noodwendig direkte negatiewe gevolge vir die hervestiging van natuurlike proteoïd struik nie, maar die nalatenskap van veranderde grondmikrobiëse gemeenskappe en die teenwoordigheid van onkruidagtige inheemse spesies kan negatiewe impakte hê op hul groei. Sekondêre indringing blyk nie habitatspesifiek te wees nie, maar word bevorder deur brand na die oorspronklike skoonmaak. En dit kan tot drie jaar voort duur op vlakke soortgelyk of hoër as die eerste jaar. Die groei van sekondêre indringers en onkruidagtige inheemse spesies neem toe met 'n toename in grondnitraatvlakke.

Ek kom tot die gevolgtrekking dat praktiserende restorasie-ekoloë grondnalatingseffekte, sekondêre indringing en onkruidagtige inheemsespesies moet bestuur nadat die indringer *A. saligna* skoongemaak is om veldherstel se uitkomst te verbeter.

Acknowledgements

I wish to express my sincere gratitude and appreciation to the following people and institutions:

My supervisors, Prof. Karen J. Esler and Dr. Mirijam Gaertner, for their invaluable support, guidance and patience. I am eternally grateful for believing in me and giving me the opportunity to learn from you.

Prof. Brian van Wilgen for motivating me to join the C·I·B and for his advice, support and guidance.

The DST-NRF Centre of Excellence for Invasion Biology and the Working for Water Programme for funding.

Prof. Dave Richardson, Dr. Sarah Davies and Dr. John Measey for their advice and support.

Suzaan Kritzinger-Klopper, Mathilda van der Vyver, Dorette Du Plessis, Erika Nortje, Rhoda Moses and Karla Coombe-Davis from the C·I·B for their help over the years. I am especially grateful to Suzaan and Erika for assisting with the translation of the abstract from English to Afrikaans.

My family and friends for supporting and encouraging me throughout this project.

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General introduction

Background information and motivation

Widespread introduction of species to areas outside of their natural distribution ranges has led some becoming invasive (Vitousek et al., 1997; Richardson et al., 2011). Many of these invasive alien species are trees and shrubs (Richardson and Rejmánek, 2011) and some are nitrogen-fixing such as Australian acacias (Richardson et al., 2011). There is evidence that invasive N₂-fixing woody species often transform ecosystems through their negative impacts on soil chemistry, seed banks and microbial communities, and native plant diversity (Richardson et al., 2000; Ehrenfeld, 2003; Richardson and Kluge, 2008; Inderjit and van der Putten, 2010; Vilà et al., 2011). Mechanisms underlying such impacts are well known (e.g. Levine et al., 2003; Vilà et al., 2011). Furthermore, the economic cost associated with invasive species across the globe is staggering (van Wilgen et al., 2001; Pimentel et al., 2005; Vilà et al., 2010). Therefore, the need to manage invasive species has been growing (Hulme, 2009).

Worldwide efforts to clear invasive species and to restore native plant diversity are currently underway (Suding et al., 2004; Le Maitre et al., 2011). It is common to assume that the negative impacts of invasive species will dissipate after clearing and lead to the recovery of native plant diversity (Wittenberg and Cock, 2005; Grove et al., 2015). However, negative impacts of invasive species can remain as legacy effects – i.e. measurable changes to biological, chemical, or physical conditions, that persist for long periods after clearing (Marchante et al., 2009; Corbin and D’Antonio, 2012; Rodríguez-Echeverría et al., 2013).

Numerous legacy effects of invasive species have been identified (reviewed by Corbin and D’Antonio, 2012). A growing number of studies describe how soil legacy effects create barriers to restoration after removal of invasive N₂-fixing woody species (Vitousek and Walker, 1989; Yelenik et al., 2004; Malcolm et al., 2008; Oneto et al., 2010; Boudiaf et al., 2013), while others present potential management actions to address these barriers to restoration (Pickart et al., 1998b; Buckley et al., 2004; DiTomaso et al., 2006; Elgersma et al., 2011; Neill et al., 2015). However, we still lack a broad overview of barriers to restoration presented by soil legacy effects of invasive N₂-fixing woody species and the management actions that could potentially be used to address them. Knowledge on barriers to restoration and their management is crucial for improving restoration outcomes.

Invasive Australian acacias form a significant proportion of global plant invasions (Richardson and Rejmánek, 2011) and are found across various habitats in different geographical regions (Le Maitre et al., 2011; Richardson et al., 2011; González-Muñoz et al., 2012). Invasion by acacias can alter soil chemistry (Musil, 1993; Marchante et al., 2008), seed banks (Richardson and Kluge, 2008), microbial community composition and function (Inderjit and van der Putten, 2010; Crisóstomo et al., 2013; Lorenzo et al., 2013), and exclude native species (González-Muñoz et al., 2012; Gaertner et al., 2012a).

Management interventions such as clearing the invasive acacias, removing their litter, reducing their seed banks, removing *Acacia* seedlings that germinate after clearing the adult plants, and active restoration through the re-introduction of native species using seed and/or vegetative propagules, are often applied to reduce their negative impacts and restore native plant diversity in previously invaded areas (Richardson and Kluge, 2008; Le Maitre et al., 2011; van Wilgen et al., 2011; Gaertner et al., 2012a, 2012b). However, it has proved difficult to successfully restore native plant diversity in previously invaded areas due to a lack of native species re-establishment (Galatowitsch and Richardson, 2005; Marchante et al., 2011).

Instead of facilitating native plant diversity recovery, clearing invasive acacias often leads to secondary invasion – i.e. an increase in the abundance of non-target alien species (Pearson et al., 2016) and weedy native species dominance – i.e. an increase in the quantity of native species that are not typically found and wanted in the target area and that have detectable impacts (Pyšek et al., 2004) in previously invaded areas (Galatowitsch and Richardson, 2005; Blanchard and Holmes, 2008; Gaertner et al., 2012b; Fill et al., 2018). Dominance of secondary invaders and weedy native species after clearing invasive acacias is often facilitated by the legacy of altered soil chemistry, particularly elevated soil nitrogen availability (Yelenik et al., 2004; Marchante et al., 2009; Le Maitre et al., 2011; González-Muñoz et al., 2012; Fill et al., 2018).

Failure of restoration efforts has been closely associated to inhibition by soil legacy effects (Macdonald, 2004; Gaertner et al., 2012b), secondary invasion (Pearson et al., 2016) and weedy native species dominance (Yelenik et al., 2004). However, limited attention has been given to understanding soil chemical and biotic legacies (see Yelenik et al., 2004; Marchante et al., 2009), secondary invasion and weedy native species dominance after clearing invasive acacias (see Yelenik et al., 2004; Galatowitsch and Richardson, 2005; Blanchard and Holmes, 2008; Gaertner et al., 2012b; Fill et al., 2018). Particularly, there is

limited knowledge on (1) how long soil chemical and biotic legacies persist after clearing invasive acacias and their effect on native plant diversity recovery; (2) the identity of secondary invaders and weedy native species after clearing invasive acacias; (3) how soil legacy effects and fire application after clearing invasive acacias affect secondary invasion and weedy native species dominance; and (4) the interactions between secondary invaders after clearing invasive acacias. To improve restoration outcomes after clearing invasive acacias, it is paramount to understand soil legacy effects, secondary invasion and weedy native species dominance.

This study used *Acacia saligna* (Labill.) H. L Wendl. (Fabaceae) invasions in the South African fynbos as case study to address these issues. Within South Africa, the fynbos biome is the greatest casualty of Australian *Acacia* invasions (van Wilgen et al., 2011). *Acacia saligna* covers approximately 53 000 ha in South Africa and a significant proportion of its distribution is in the fynbos (Van Wilgen et al., 2011).

Aims and objectives of the study

This study had six aims and objectives (Figure I) with the end goal of providing management recommendations to improve restoration outcomes. The first aim was to understand how soil legacy effects of invasive alien N₂-fixing woody species present barriers to restoration and identify management actions that could potentially be used to address these barriers to restoration through a global literature review. The second aim was to determine how long soil legacy effects of invasive *A. saligna* persist after clearing through soil sample analyses. The third aim was to investigate the effect of invasive *A. saligna*'s soil chemical and biotic legacies, and weedy native species on native species re-establishment through a greenhouse experiment. The fourth aim was to identify species that are secondary invaders after clearing invasive *A. saligna* and investigate the effects of vegetation type, and fire application on their establishment over three years after clearing through vegetation sampling. The fifth aim was to investigate the interactions between secondary invaders and the extent to which soil nitrate levels, apparent after clearing invasive *A. saligna*, influence secondary invasion and weedy native species dominance through a greenhouse experiment. By synthesising the knowledge gained from this study, management recommendations to improve restoration outcomes are presented. Further details of each chapter are provided in the chapter synopses below.

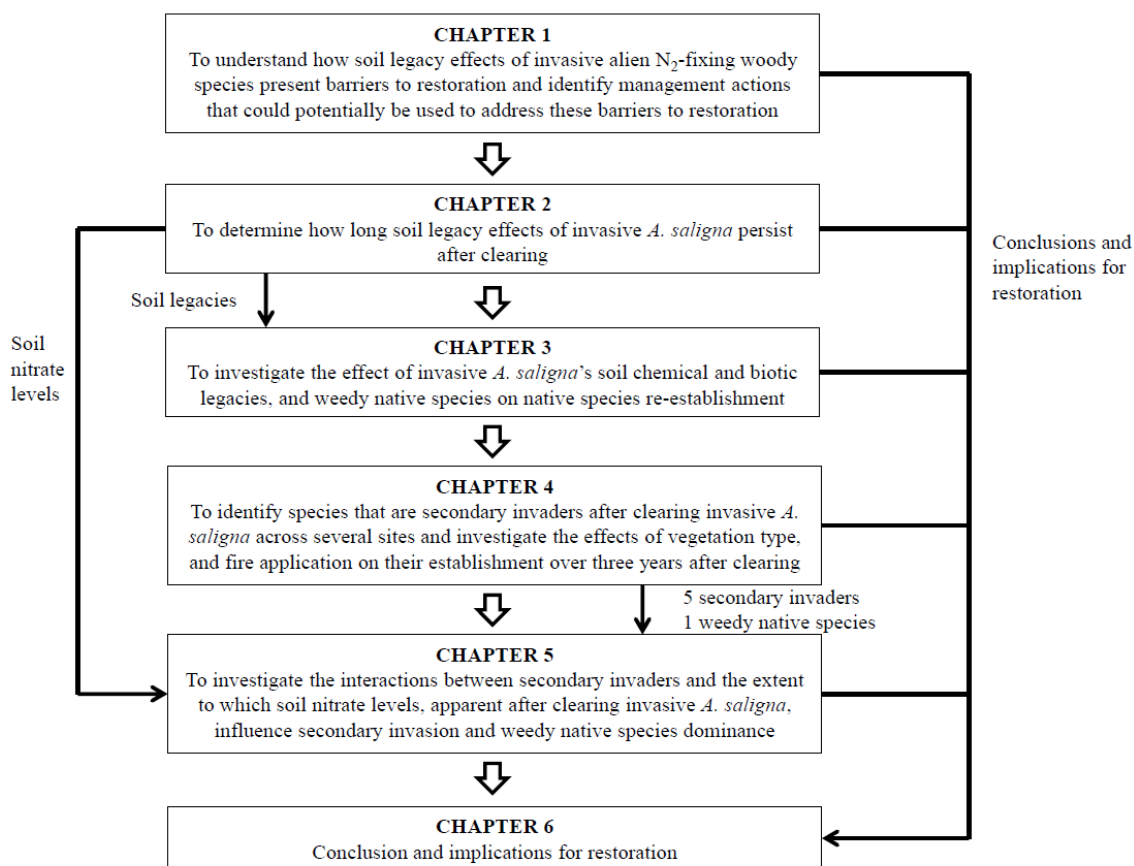


Figure I: The aims of each chapter and how they link together within the study.

Chapter synopses

This section provides a short synopsis of each chapter including where it was published or is intended for submission and the relative contributions of different authors.

Chapter one: Barriers to ecosystem restoration presented by soil legacy effects of invasive alien N₂-fixing woody species: implications for ecological restoration

Reference: Nsikani, M.M., van Wilgen, B.W., Gaertner, M., 2018. Barriers to ecosystem restoration presented by soil legacy effects of invasive alien N₂-fixing woody species: implications for ecological restoration. *Restoration Ecology* 26, 235–244.

This chapter presents a global review of how soil legacy effects of invasive alien N₂-fixing woody species present barriers to restoration, and management actions that could potentially be used to address them. This chapter found that altered soil microbial communities, depleted native soil seed banks, elevated N status, secondary invasion and weedy native species dominance, and reinvasion are potential barriers to restoration.

Furthermore, carbon addition, litter removal, soil microbial treatments, establishing species adapted to low N levels, prescribed burning, classical biological control, grazing, mowing, herbicide or graminicide application, manual weeding, soil N management, soil solarization, weed mats, native species reintroduction, and nurse plants are potential management actions for these barriers to restoration. This chapter found that management actions are rarely applied in combination, despite that they often address distinct barriers to restoration. Therefore, this chapter recommends that management actions should be combined into an integrated management effort to improve restoration outcomes. Mr. Mlungele M. Nsikani and Dr. Mirijam Gaertner designed the review. Mr. Mlungele M. Nsikani conducted the review and wrote the chapter. Prof. Brian van Wilgen and Dr. Mirijam Gaertner made comments to improve it.

Chapter two: *Acacia saligna*'s soil legacy effects persist up to 10 years after clearing: Implications for ecological restoration

Reference: Nsikani, M.M., Novoa, A., van Wilgen, B.W., Keet, J.H., Gaertner, M., 2017. *Acacia saligna*'s soil legacy effects persist up to 10 years after clearing: Implications for ecological restoration. *Austral Ecology* 42, 880–889.

This chapter examines how long soil legacy effects of invasive *A. saligna* persist after clearing. Differences in soil chemical characteristics and enzyme activities between invaded, cleared (i.e. two, six and ten years after clearing) and non-invaded areas were determined. This chapter presents evidence that invasion by *A. saligna* alters overall soil characteristics. Moreover, soil characteristics are not restored to natural conditions after clearing (soil legacy effects persist up to ten years after clearing). Moreover, clearing invasive *A. saligna* elevates soil nitrate levels and these can remain higher than in invaded and non-invaded areas up to ten years after clearing. This chapter recommends that active restoration by planting native species could, over time, return soils to natural conditions. Mr. Mlungele M. Nsikani and Dr. Mirijam Gaertner designed the research. Mr. Mlungele M. Nsikani, Dr. Ana Novoa and Mr. Jan-Hendrik Keet collected and analysed the data. Mr. Mlungele M. Nsikani wrote the chapter. Dr. Ana Novoa, Prof. Brian van Wilgen, Mr. Jan-Hendrik Keet and Dr. Mirijam Gaertner made comments to improve it.

Chapter three: Re-establishment of *Protea repens* after clearing invasive *Acacia saligna*: Consequences of soil legacy effects and a native nitrophilic weedy species

Reference: Nsikani, M.M., van Wilgen, B.W., Bacher, S., Gaertner, M., 2018. Re-establishment of *Protea repens* after clearing invasive *Acacia saligna*: Consequences of soil legacy effects and a native nitrophilic weedy species. *South African Journal of Botany* 116, 103–109.

This chapter examines the effect of invasive *A. saligna*'s soil chemical and biotic legacies, and weedy native species on native species re-establishment. *Protea repens* (L.) L (Proteaceae) was grown with or without *Ehrharta calycina* (Sm.) (Poaceae) in sterilized and non-sterilized soil collected from the same cleared and non-invaded areas used in chapter two. This chapter presents evidence that the legacy of altered soil chemistry after clearing invasive *A. saligna* does not necessarily have direct negative consequences on the re-establishment of native proteoid shrubs. Furthermore, while soil microbial communities after clearing invasive *A. saligna* may have a positive effect on the germination of native proteoid shrubs, the legacy of altered soil microbial communities and presence of weedy native species could have negative impacts on their growth. This chapter recommends that restoration efforts do not always have to include management of altered soil chemistry after clearing invasive *A. saligna*. Mr. Mlungu M. Nsikani and Dr. Mirijam Gaertner designed the research. Mr. Mlungu M. Nsikani conducted the greenhouse experiments and collected the data. Mr. Mlungu M. Nsikani and Prof. Sven Bacher analysed the data. Mr. Mlungu M. Nsikani wrote the chapter. Prof. Brian van Wilgen, Prof. Sven Bacher and Dr. Mirijam Gaertner made comments to improve it.

Chapter four: Secondary invasion after clearing invasive *Acacia saligna* in the South African fynbos

This chapter is intended for submission to the *South African Journal of Botany*

This chapter identifies species that are secondary invaders after clearing invasive *A. saligna* and explores the effects of vegetation type, and fire application on their establishment over three years after clearing. Lowland and mountain fynbos vegetation was monitored in and outside of burn scars – i.e. areas in which *Acacia* biomass was stacked and burnt, for three years after clearing invasive *A. saligna* using the “fell, stack and burn” method – i.e. a management method that involves felling primary invaders, stack the slash and allow it to dry

before burning it. This chapter presents evidence that up to 32 species can be secondary invaders after clearing invasive *A. saligna*. Furthermore, application of fire after clearing invasive *A. saligna* appears to favour the dominance of secondary invaders. Secondary invasion appears not to be habitat specific – i.e. occurs in both lowland and mountain fynbos, and can persist up to three years after clearing at levels similar to or higher than the first year. This chapter calls for the management of secondary invasion through actions such as herbicide or graminicide application, grazing, manual weeding, mowing, prescribed burning, soil nitrogen management, soil solarization and weed mats to improve restoration outcomes. Mr. Mlungu M. Nsikani, Dr. Mirijam Gaertner and Prof. Karen J. Esler designed the research and collected data. Mr. Mlungu M. Nsikani analysed the data and wrote the chapter. Dr. Mirijam Gaertner and Prof. Karen J. Esler made comments to improve it.

Chapter five: Soil nitrogen availability and competitive interactions shape secondary invasion and weedy native species dominance after clearing invasive *A. saligna*

This chapter is intended for submission to the *South African Journal of Botany*

This chapter explores the interactions between secondary invaders and the extent to which soil nitrate levels after clearing invasive *A. saligna* influence secondary invasion and weedy native species dominance. Four secondary invaders and one weedy native species identified in chapter four were germinated and grown in the absence of interspecific competition in the highest, median and lowest soil nitrate levels from cleared areas used in chapter two. Additionally, two of these secondary invaders were germinated and grown in monocultures and mixtures in varying seed or plant densities. This chapter presents evidence that growth of secondary invaders and weedy native species decreases with a decline in soil nitrate levels. However, some secondary invaders and weedy native species grow better than others at the same soil nitrate level. Furthermore, despite similarity in growth form, some secondary invaders are less competitive than others such that they grow better in monocultures than mixtures. Therefore, some secondary invaders can exclude others in mixtures. This chapter recommends the management of soil nitrogen availability after clearing invasive *A. saligna* reduce secondary invasion and weedy native species dominance and improve restoration outcomes. Mr. Mlungu M. Nsikani, Dr. Mirijam Gaertner and Prof. Karen J. Esler designed the research. Mr. Mlungu M. Nsikani conducted the greenhouse experiments, collected and analysed the data, and wrote the chapter. Dr. Mirijam Gaertner and Prof. Karen J. Esler made comments to improve it.

Chapter six: Conclusion and implications for restoration.

This chapter summarises the major findings of this study and recommends management actions to improve restoration outcomes after clearing invasive *A. saligna*. Mr. Mlungu M. Nsikani wrote the chapter. Dr. Mirijam Gaertner and Prof. Karen Esler made comments to improve it.

Chapter one: Barriers to ecosystem restoration presented by soil legacy effects of invasive alien N₂-fixing woody species: implications for ecological restoration

Please use the link below to access the full list of appendices:

<https://onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1111%2Frec.12669&file=rec12669-sup-0001-AppendixS1-S3.docx>

1.1 Abstract

Impacts of invasive alien N₂-fixing woody species and how they can persist as soil legacy effects after invasive species control are well appreciated, but how soil legacy effects can present barriers to restoration is poorly understood. Finding better ways to deal with these barriers to restoration is essential to improving restoration outcomes. In this study, we review 440 studies to identify barriers to restoration and potential management actions for the barriers to restoration, and provide practical application examples of the management actions. Our findings suggest that altered soil microbial communities, depleted native soil seed banks, elevated N status, secondary invasion and weedy native species dominance, and reinvasion are potential barriers to restoration. Furthermore, carbon addition, litter removal, soil microbial treatments, establishing species adapted to low N levels, prescribed burning, classical biological control, grazing, mowing, herbicide or graminicide application, manual weeding, soil N management, soil solarization, weed mats, native species reintroduction, and nurse plants are potential management actions for these barriers to restoration. However, there is little evidence suggesting that several of these barriers to restoration hinder improved restoration outcomes and this could be due to little research on them. More research is needed to assess their relative importance in hindering improved restoration outcomes. Management actions are rarely applied in combination, despite that they often address distinct barriers to restoration. Management actions should be combined into an integrated management effort to improve restoration outcomes.

1.2 Introduction

The widespread introduction of plant species to areas outside of their natural distribution ranges has led to some becoming invasive (Vitousek et al., 1997). Many of these invasive alien species are trees and shrubs (Richardson and Rejmánek, 2011) and some are nitrogen-fixing such as Australian acacias (Richardson et al., 2011). Invasive N₂-fixing woody species often transform ecosystems by altering ecosystem processes and displacing native species

(Vilà et al., 2011). The negative impacts of invasive N₂-fixing woody species on the soil include altered soil chemistry (Ehrenfeld, 2003), establishment of large seed banks (D'Antonio and Meyerson, 2002; Richardson and Kluge, 2008), deposition of novel allelochemicals (Callaway and Ridenour, 2004), and altered soil microbial community composition and function (Inderjit and van der Putten, 2010). Mechanisms underlying such impacts are well documented (e.g. Levine et al., 2003; Vilà et al., 2011).

Worldwide efforts are underway to clear invasive species and restore historical ecosystems (D'Antonio and Meyerson, 2002; Suding et al., 2004). It is often assumed that negative impacts of invasive species will diminish after clearing (Wittenberg and Cock, 2005), but this is not always the case because the invasive species can leave persistent legacy effects (i.e. measurable changes to biological, chemical, or physical conditions) in the soil (Corbin and D'Antonio, 2012). Ecophysiological traits of invasive N₂-fixing woody species (e.g. early and high production of seeds with long dormancy periods, high growth rates, and increasing the N content of N-limited soil) contribute to the persistence of soil legacy effects after their clearing (Pyšek and Richardson, 2007). Numerous legacy effects of invasive species have been identified (reviewed by Corbin and D'Antonio, 2012). Restoration of historical ecosystems hence often fails after invasive species clearing, probably because soil legacy effects create barriers to restoration (Corbin and D'Antonio, 2012).

A growing number of studies describe how soil legacy effects present barriers to restoration after removal of invasive N₂-fixing woody species (Appendix S2) while others present potential management actions to address these barriers to restoration (Appendix S3). Efforts have been made to review potential management actions for these barriers to restoration (e.g. Perry et al., 2010), but these have only focused on management of individual barriers to restoration. We still lack a broad review of barriers to restoration presented by soil legacy effects and management actions that could potentially be used to address them. In this study, we review (1) how soil legacy effects present barriers to restoration after invasive N₂-fixing woody species clearing; (2) potential management actions to address those barriers to restoration; and (3) we give practical examples of their application. We discuss all soil legacy effects of invasive alien N₂-fixing woody species, not just those directly related to N₂-fixation and elevated soil N to maximize the usefulness of the review to restoration practice.

Table 1.1: Combinations of search terms designed to locate studies that document barriers to restoration presented by soil legacy effects of invasive alien N₂-fixing woody species and potential management actions to address them.

<i>Barriers to restoration and potential management actions specific to invasive N₂-fixing woody species</i>	<i>Potential management actions for barriers to restoration that also apply to invasive non-N₂-fixing woody species</i>	<i>Potential management actions for each identified barrier to restoration</i>
exotic* OR invasive* OR invasion* OR alien* OR invader* OR non-native* OR nonnative* AND nitrogen-fix* OR nitrogen fix* OR dinitrogen-fix* OR dinitrogen fix* OR N-fix* OR N fix* OR N2-fix* OR N2 fix* OR actinorhizal* OR legume* OR leguminous* OR root nodule* AND impact* OR effect* OR legacy* OR legacies* OR legacy effect* AND native* OR indigenous* OR restoration* OR recovery* OR reestablishment* OR re-establishment* OR return* OR management*	exotic* OR invasive* OR invasion* OR alien* OR invader* OR non-native* AND legac* OR residual* OR long lasting* AND restor* OR recover* OR return* OR manage*	seed bank* OR seedbank* OR microbe* OR reinvasion* OR re-invasion* OR secondary invad* OR ruderal* OR soil nitrogen* AND native* OR indigenous* OR restor* OR recover* OR return* OR manage* OR reestablishment* OR re-establishment*

Table 1.2: Potential management actions to address barriers to restoration presented by soil legacy effects of invasive alien N₂-fixing woody species.

<i>Management action</i>	<i>Barrier to restoration</i>				
	<i>Elevated soil N status</i>	<i>Secondary invaders and weedy native species</i>	<i>Re-invasion</i>	<i>Depleted native soil seed banks</i>	<i>Altered soil microbial communities</i>
Soil carbon addition	X				
Litter removal	X				X
Soil microbial treatments					X
Establishing species adapted to low N availability	X				
Prescribed burning	X	X	X		
Classical biological control			X		
Grazing		X	X		
Mowing		X	X		
Herbicide or graminicide application		X	X		
Manual weeding		X	X		
Soil N management		X			
Soil solarization		X	X		
Weed mats		X	X		
Native species re-introduction				X	
Nurse plants				X	

1.3 Methods

We searched for relevant articles on the ISI Web of Science database (<http://www.webofknowledge.com>) with no restriction on publication year, using a range of keywords (Table 1.1) designed to locate articles that document barriers to restoration presented by soil legacy effects of invasive alien N₂-fixing woody species and their management. Abstracts of retrieved articles were read and those relevant to this study were selected and the full paper content read.

1.4 Results

We identified 440 articles (35 on barriers to restoration, 399 on management actions, and 6 discussing both aspects; Appendix S1) that were relevant to this study. Some of those articles were reviews (e.g. Le Maitre et al., 2011) that included studies from the “gray literature”; therefore, we achieved a reasonably good coverage of the literature on barriers to restoration and their management, not restricted to that indexed in the Web of Science. We identified that elevated N status, secondary invasion and weedy native species dominance, reinvasion, depleted native soil seed banks, and altered soil microbial communities have all been noted as barriers to restoration following clearing of invasive, N₂-fixing woody species (Figure 1.1). We further identified that carbon addition, litter removal, soil microbial treatments, establishing species adapted to low N levels, prescribed burning, classical biological control, grazing, mowing, herbicide or graminicide application, manual weeding, soil N management, soil solarisation, weed mats, native species reintroduction, and the use of nurse plants are methods that have been used to manage these barriers to restoration (Figure 1.2).

1.5 Barriers to restoration

The subject of barriers to restoration presented by soil legacy effects of invasive N₂-fixing woody species has not been extensively reported in the literature as shown by the small number (41) of publications selected for this review. Many important concepts are described by relatively few studies, with a strong bias towards certain regions – e.g. the United States (Appendix S2). While more, and as yet unidentified, barriers to restoration may exist, we believe that the few available studies have allowed us to develop a fairly robust picture of how legacy effects of invasive N₂-fixing woody species could present barriers to restoration. Each of the barriers to restoration is described in the following subsections.

Elevated Soil N Status. Invasive N₂-fixing woody species generally increase soil N through N₂-fixation and production of N-rich litter (Vitousek and Walker, 1989; Malcolm et al., 2008). After clearing, elevated N content and availability, and altered N mineralization, can persist and increase further with decomposition of invader litter and roots that remain. Alterations to soil N status are often measured through soil sample analysis by comparing non-invaded, invaded, and/or previously invaded sites (Yelenik et al., 2004; Von Holle et al., 2013). Native species in some habitats are adapted to low soil N – e.g. pine-oak systems in the United States; hence, persistent elevated soil N status can directly hinder restoration by negatively affecting their germination, growth, diversity, and/or indirectly hinder restoration by giving a competitive advantage to weedy species adapted to high N availability (Rice et al., 2004).

Secondary Invasion and/or Weedy Native Species Dominance. Seeds of alien or native nitrophilous species can be present in the soil seed bank of restoration sites or disperse to such sites from surrounding areas (Yelenik et al., 2004; Pearson et al., 2016). These nitrophilous species are often more competitive than native restoration species under high N conditions (Pearson et al., 2016). Such species often take advantage of the conditions created by removing invasive N₂-fixing woody species to establish (Pearson et al., 2016). Moreover, the elevated soil N status created by the invasive N₂-fixing woody species facilitates such species' growth and dominance (Vitousek and Walker, 1989). Secondary invaders and weedy native species have been observed to hinder restoration by limiting the growth of native restoration species (Maron and Connors, 1996; Yelenik et al., 2004; Marchante et al., 2009; Von Holle et al., 2013).

Reinvasion. Invasive N₂-fixing woody species often produce copious amounts of seed that can persist in the seed bank for extended periods (Oneto et al., 2010). This often leads to germination and reinvasion after clearing. Furthermore, clearing the invasive species often leaves roots or stumps of the plants, which can resprout in some cases (MacDonal and Wissel, 1992; Shortt and Vamosi, 2012; Souza-Alonso et al., 2013). Reinvasion of restoration sites (through persistent seed and/or vegetative propagules) allows the invasive species to once again dominate the ecosystem, leading to failed restoration (Holmes and Cowling, 1997b).

Depleted Native Soil Seed Banks. Invasive N₂-fixing woody species often compete with and exclude native species (Vilà et al., 2011). The exclusion of native species can lead to depleted native soil seed banks – due to native species becoming greatly reduced in numbers, and with survivors producing less seed as they either do not reach maturity or do not flower under the canopy of the invader. After clearing the invasive species, the depleted native soil seed banks often become a barrier to reestablishment of native communities (Malcolm et al., 2008).

Altered Soil Microbial Communities. Invasive N₂-fixing woody species can alter the soil microbial community composition, diversity, and function through several mechanisms such as deposition of allelochemicals and introduction of novel microbes (Inderjit and van der Putten, 2010). The soil mycorrhizal community and symbioses of native species are often disrupted and such changes can persist after clearing the invasive species and limit the germination and/or growth of native species (Corbin and D’Antonio, 2012; Boudiaf et al., 2013).

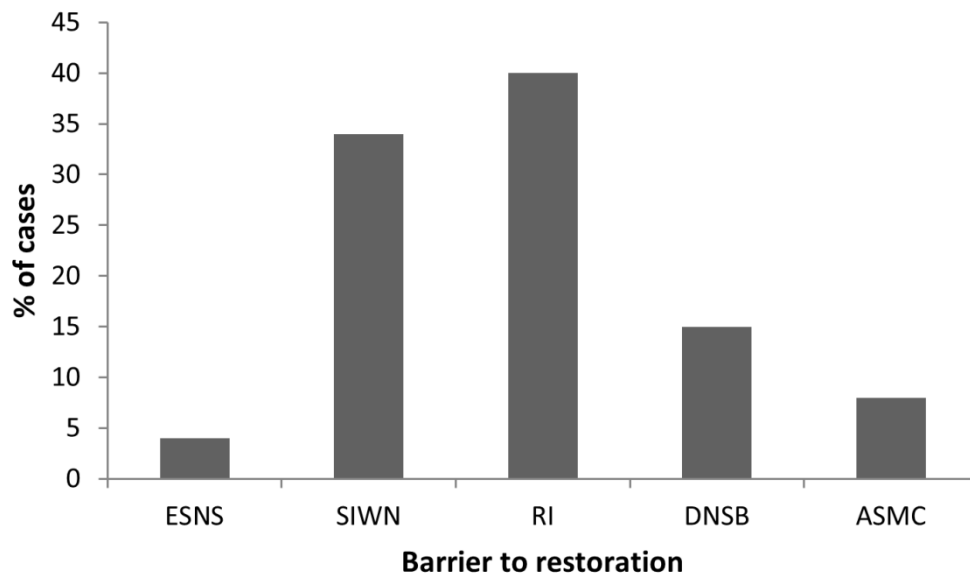


Figure 1.1: Percentage of cases (n= 53) identified from 41 studies describing barriers to restoration presented by soil legacy effects of invasive alien N₂-fixing woody species included in this review. ESNS: elevated soil N status; SIWN: secondary invaders and weedy native species; RI: re-invasion; DNSB: depleted native species’ soil seed banks; ASMC: altered soil microbial communities.

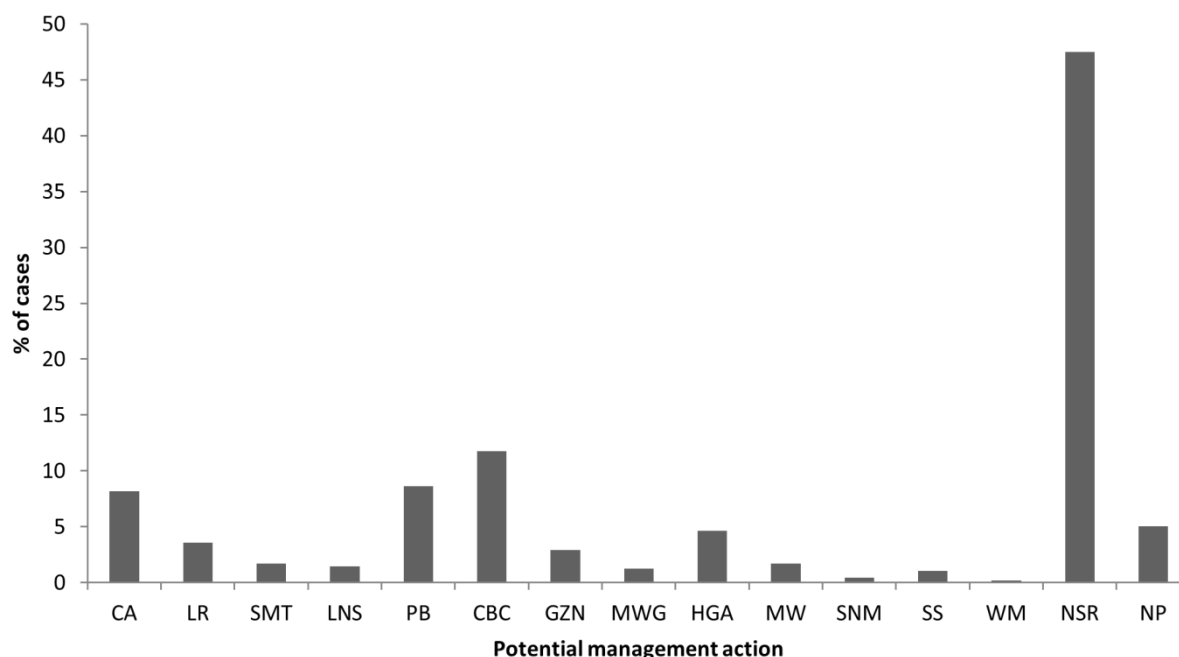


Figure 1.2: Percentage of cases (n = 476) identified from 405 studies describing potential management actions to address barriers to restoration presented by soil legacy effects of invasive alien N₂-fixing woody species included in this review. CA: carbon addition; LR: litter removal; SMT: soil microbial treatments; LNS: establish species adapted to low N levels; PB: prescribed burning; CBC: classical biological control; GZN: grazing; MWG: mowing; HGA: herbicide or graminicide application; MW: manual weeding; SNM: soil N management; SS: soil solarization; WM: weed mats; NSR: native species re-introduction; NP: nurse plants.

1.6 Management of barriers to restoration

These barriers to restoration can be addressed using a range of appropriate management actions (Table 1.2). Potential management actions for different barriers to restoration are described and illustrated using selected examples below.

Elevated Soil N Status. Prescribed burning can be used to remove the invasive species' litter to prevent it from contributing to the soil N pool in the long term (Mitchell et al., 2000). The slash can be spread over the restoration site and burnt, instead of being stacked before burning (DiTomaso et al., 2006). Prescribed burning will initially cause a strong pulse of released N previously immobilized in the litter (Fenn et al., 1998). A significant portion of the released N will be volatilized (Riggan et al., 1994; Marchante et al., 2009), whereas released NH₄⁺ will probably be nitrified after burning and result in leached NO₃⁻ (Dunn et al.,

1979). Repeated burning can deplete soil N through repeated volatilization (Prober et al., 2005). For example, in grasslands of the United States repeated burning reduced total N by up to 40% in the top 10 cm of the soil (Haubensak et al., 2004). There is a need to consider the effects of fire on restoration sites because the application of fire may be inappropriate for some habitat types – e.g. riparian zones (Blanchard and Holmes, 2008; Richardson and Kluge, 2008). Furthermore, there is a need to consider the effects of fire on native and invasive species' seed banks as this will ultimately affect native plant diversity recovery. In areas where the use of fire is inappropriate, manual techniques such as raking can be used to remove the invasive species' litter (Elgersma et al., 2011). However, such techniques are labor intensive and logistically limited to small areas.

Labile carbon sources such as sawdust and sucrose can be added or leached into the soil to immobilize soil N (Blumenthal et al., 2003; Prober and Thiele, 2005). Briefly, the added carbon increases soil microbial biomass and activity – by serving as a substrate for heterotrophic soil microbes, which in turn increases microbial N uptake and lowers soil N availability (Baer et al., 2003). Furthermore, carbon addition in anaerobic soils can increase the activity of denitrifying bacteria and increase N loss through denitrification (Perry et al., 2010). For example, in a coastal sage scrub in the United States, addition of organic mulch resulted in a significant decrease in soil N availability (Zink and Allen, 1998). Success of carbon addition depends on whether or not soil microbes are C-limited, method and duration of application, environmental conditions, habitat type, and target species (Rice et al., 2004; Prober and Thiele, 2005), and its long-term effects are often difficult to predict (Török et al., 2014). Furthermore, the success of carbon addition may be hindered because it is labor intensive and expensive to apply at large scale (Perry et al., 2010).

Many species adapted to low N availability can lower soil N because they produce high C:N litter, which slows N cycling, and they have lower minimum N requirements, which allow them to continue to grow and capture more limited available N (Perry et al., 2010). Therefore, establishing species adapted to low N availability in the site designated for restoration can ultimately lower soil N (Perry et al., 2010). For example, the establishment of native *Themeda australis* in temperate grassy woodlands significantly reduced soil N availability (Prober et al., 2005). Native restoration species in areas invaded by N₂-fixing woody species are often adapted to low N availability; hence, waiting for them to establish from their soil seed banks, seeding, or transplanting them (i.e. if soil seed banks are depleted) in restoration sites may be appropriate. Furthermore, use of nurse plants adapted to low N

availability during native species reintroduction could potentially lower soil N. However, establishment of species adapted to low N availability may not significantly lower soil N over short time scales or when certain N levels have been reached (Perry et al., 2010). Repeated prescribed burning can favor species adapted to low N availability (Perry et al., 2010). For example, in an oak savanna in the United States, repeated prescribed burning led to a shift from oak to grass dominance and a subsequent decline in soil N levels due to low tissue N concentrations in grasses (Dijkstra et al., 2006).

Secondary Invasion and Weedy Native Species Dominance. Establishment of secondary invaders and weedy native species can be reduced through soil solarization (i.e. heating the soil surface by covering with a plastic sheet) or setting up weed mats (i.e. woven plastic mats that allow passage of water but prevent emergence of seedlings) in restoration sites. For example, weed mats set up in a coastal dune cleared of *Lupinus arboreus* reduced the establishment of secondary invaders (Pickart et al., 1998b). However, the cost and logistical challenges restrict the use of soil solarization or weeds mats to small areas (Pickart et al., 1998b; Richardson and Kluge, 2008). Furthermore, there is need to consider the extent and effect of soil solarization and weed mats on native species because these techniques are not species specific and could hinder native plant diversity recovery. Secondary invaders and weedy native species that establish can be manually weeded, mowed, selectively grazed (Maron and Jefferies, 2001; Gooden et al., 2009; Milchunas et al., 2011), or controlled through herbicide or graminicide application (Sztár et al., 2016). For example, mowing followed by biomass removal during restoration of coastal prairie grasslands reduced the abundance of secondary invaders (Maron and Jefferies, 2001). If grazing or mowing are used to control secondary invaders and weedy native species, season, intensity, and duration of application need to be considered to obtain satisfactory results and avoid negative ecological consequences (Milchunas et al., 2011; Dee et al., 2016). Appropriate herbicides or graminicides should be selected and proper timing of their application, toxicity, residence times, and specificity should be carefully considered (Hobbs and Humphries, 1995; Oneto et al., 2010). The technique used to apply the herbicide or graminicide would depend on the size of the restoration site – e.g. broadcast foliar treatment for large areas and drizzle technique for small areas (Oneto et al., 2010).

Invasive alien and weedy native species often prefer high N availability (D'Antonio and Meyerson, 2002; Pearson et al., 2016); hence, their dominance can decline if elevated soil N is addressed with soil N management, as described above (Kulmatiski, 2011). For example,

reduction of N availability after the death of *L. arboreus* significantly reduced the abundance of secondary invaders (Alpert and Maron, 2000).

Reinvasion. To address reinvasion by N₂-fixing, invasive alien plants, classical biological control can be set up when available to reduce seed production and seed banks over time (Buckley et al., 2004). For example, long-term classical biological control of invasive *Acacia* spp. in South Africa using nine insect species and a fungus has led to significant reductions in their seed production, seed banks, and distribution (Moran and Hoffmann, 2012). However, it should be noted that biological control is not a quick-fix solution to reduce persistent seed banks (Moran and Hoffmann, 2012). Furthermore, predation rate of biological control agents should be high (>90%) for classical biological control to be effective (Noble and Weiss, 1989). Biological control agents often take time to establish but once established they can be efficient in reducing fecundity of invasive species by destroying their flowers, buds, or pods (Holmes and Cowling, 1997b; Buckley et al., 2004).

Numerous invasive species accumulate persistent soil seed banks characterized by seeds that need a heat pulse to break dormancy (Richardson and Kluge, 2008). Therefore, persistent soil seed banks can be reduced by triggering mass germination through prescribed burning using low-intensity fires after clearing the invasive species (Holmes and Cowling, 1997b). Use of low-intensity fires is recommended because high-intensity fires tend to destroy native seeds and seedlings that may be present on restoration sites (Richardson and Kluge, 2008). Furthermore, burning will kill a significant part of the invasive species' seed on the soil surface and litter (DiTomaso et al., 2006). Seedlings that germinate can be manually weeded (Fill et al., 2017), treated with herbicides (Krupek et al., 2016), and mowed or selectively grazed (Richardson and Kluge, 2008) to avoid the development of a second generation of dense invasive species. Multiple treatments may be required to achieve desired effects (Mandle et al., 2011). For example, a combination of prescribed burning and herbicide application after invasive *Acacia mearnsii* control in South Africa significantly reduced its soil seed banks (Campbell et al., 1999).

Soil solarization and weed mats could be viable alternatives to prescribed burning in sites where use of fire is inappropriate. For example, soil solarization treatments substantially reduced the number of buried seeds of *A. saligna*, *A. murrayana*, and *A. sclerosperma* in Israel (Cohen et al., 2008). Weed mats set up in a coastal dune in the United States reduced

reinvansion of *L. arboreus* (Pickart et al., 1998b). However, the use of soil solarization and weed mats is logistically and financially limited to small areas (Pickart et al., 1998b; Richardson and Kluge, 2008).

Depleted Native Soil Seed Banks. Native species reintroduction through seed, vegetative propagules, transfer of seed containing plant material, or native topsoil translocation is a viable option to manage depleted native soil seed banks (Holmes and Cowling, 1997b; Baasch et al., 2012; Ferreira and Vieira, 2017). For example, seeding with native species during restoration of coastal sandplain grassland in the United States increased native species diversity (Neill et al., 2015). There is need for careful planning and clearly defined restoration goals before conducting native species reintroduction (Honnay et al., 2002; Szitár et al., 2016). For example, some restoration programs might focus on rehabilitation of functional groups or clusters of focal species, whereas others might focus on particular endangered species (Palmer et al., 1997). Practicing restoration ecologists should consider the native species to be reintroduced, donor sites to be used, timing, order, and methods of reintroduction, seed mixtures, and seeding rates for each species.

To the extent possible, timing, order, and methods of reintroduction should be informed by ecological community theory, based on known patterns of community structure (Zedler, 2000). This might involve, for example, mimicking natural successional processes by introducing early-successional or mid-successional species first, to help create suitable conditions for later-successional species introduced later (Lithgow et al., 2013). Alternatively, it could involve introducing rare species first to make sure they are not excluded by more common, rapidly establishing species (Palmer et al., 1997). Seed mixtures should be site specific and carefully selected, but diverse seed mixtures are often preferred because they offer insurance that some species will establish (Kiehl et al., 2010). Seeding rates are difficult to gauge, being species and site specific. Therefore, if available, practicing restoration ecologists should select seeding rates according to reference sites (Holmes and Richardson, 1999).

Seeds should be harvested from nearby areas to match the genetic composition of native species that occupied the restoration site before invasion (Schaefer, 2011). Furthermore, seeds should be harvested from multiple source populations to increase genetic variability (Ödman et al., 2012). Consideration should be made on the seed ecology of the native species to be reintroduced so that seeds of best quality are collected at the right time and stored

appropriately to maintain their viability (Holmes and Richardson, 1999). To improve germination, pre-treatment of native species' seeds that require certain conditions to break dormancy should be conducted before sowing (Neill et al., 2015). Seeds should be sown using an appropriate method (e.g. mechanical broadcast seeding) at a time that coincides with ideal conditions for germination and survival (Carrick and Krüger, 2007). Care should be taken to cover the seeds with substrate to reduce seed predation and prevent them from blowing away (Pausas et al., 2004).

If available, seed containing plant material (e.g. hay and litter) can be harvested from multiple native sites, transferred, and spread on the site designated for restoration as a seed source (Kiehl et al., 2006). Some species such as late successional and endangered species can be transplanted to the site designated for restoration after being grown in pots to improve their chances of establishment (Manchester et al., 1999). If native topsoil becomes available due the native site being destroyed, it can be collected (depth of 10 cm), transferred and thinly spread on the site designated for restoration as a seed source (Rokich et al., 2000). The topsoil should not be stockpiled, but spread as soon as possible to maintain viability of seeds in the soil (Rokich et al., 2000). Disturbance during topsoil translocation may be enough to break seed dormancy, but the soil can also be treated with smoke water solution to break the dormancy of some seeds (Rokich et al., 2002). However, not all seeds will germinate (Klimeš et al., 2010). Furthermore, topsoil translocation can damage species that reproduce through vegetative means (Craig and Buckley, 2013).

Nurse plants can be planted before or together with the native species to be reintroduced to create safe sites with favorable microclimates for germination and survival of the reintroduced native species (Padilla and Pugnaire, 2006). Nurse plants can shade the reintroduced native species, reduce solar radiation and soil temperatures, protect from herbivore damage, and/or improve the water status of the target seedlings by reducing evaporation (Aerts et al., 2007). For example, use of shrubs as nurse plants enhanced growth of native conifers during reforestation in Spain by improving seedling water status, and therefore reducing seedling mortality by drought (Castro et al., 2002). Species that can be used as nurse plants should have a facilitative effect on the native restoration species (Gómez-Ruiz et al., 2013). It is advisable to use short-lived species as nurse plants, so that they exist for a limited period and eventually give way to the native restoration species (Gómez-Aparicio, 2009). Also, nurse plants planted at too high densities may compete with and exclude target species (Paz Esquivias et al., 2015).

Altered Soil Microbial Communities. Practicing restoration ecologists should consider determining the extent of changes to microbial communities in the site designated for restoration (e.g. using the analysis of phospholipid fatty acids method to compare microbial communities to reference sites; Frostegård et al., 2011), to inform the selection of appropriate management actions to aid recovery of microbial communities. Soil microbial treatments can be utilized to aid the recovery of microbial communities (Richter and Stutz, 2002). For example, application of fungicides, bactericides, and microbial and fungal inoculants had a positive impact on the recovery of soil microbial communities and various native plant species in an experimental study in the United States (Perkins and Hatfield, 2016). However, the success of efforts to restore soil microbial communities has been inconsistent, context-dependent, and logistically limited to small areas (Perkins and Hatfield, 2016). Restoration of native species in the site designated for restoration could promote arbuscular mycorrhizal fungi proliferation and diversity (Tanner and Gange, 2013). Removal of invasive species' litter in the site designated for restoration (through prescribed burning or manually) could be an easier way to rectify altered soil microbial communities (Elgersma et al., 2011). However, changes to microbial communities after litter removal are often minimal and unpredictable (Elgersma et al., 2011).

1.7 The way forward

Soil legacy effects can present several barriers to restoration after clearing invasive N₂-fixing woody species (Figure 1.1). The unique N₂-fixing ability of these invasive species contributes directly to elevated N status, secondary invasion and weedy native species dominance, and altered soil microbial communities. Only a few studies in our review provided evidence that elevated N status, depleted native soil seed banks, and altered soil microbial communities are common barriers to restoration, but this does not necessarily indicate that these barriers to restoration are unimportant. Instead, we believe that the low number of reported cases for some barriers to restoration is a result of these barriers to restoration not being investigated during restoration programs. An investigation of these barriers, however, is crucial to gain a better understanding of the mechanisms of barriers to restoration following invasive species control and of what actions are most needed to improve restoration outcomes.

Management of barriers to restoration is often necessary to improve restoration outcomes and a wide array of potential approaches is available (Figure 1.2). We concede that

management actions included in this review cannot be uniformly applied in every site designated for restoration because their selection is often context-dependent (e.g. habitat type, site conditions, and scale). However, we believe that the low numbers of reported cases for many of the management approaches is a result of practicing restoration ecologists not applying them even when necessary and appropriate for managing barriers to restoration.

There is a need for restoration ecologists to investigate all potential barriers to restoration during restoration programs. Furthermore, given that they are appropriate in their context, we encourage restoration ecologists to apply the management actions included in this review. Also, we encourage the publication of results (even if they are negative) obtained when investigating all potential barriers to restoration and applying management actions included in this review.

Management actions were often applied individually. Only 25 out of 405 studies that discussed potential management actions used more than one management action (Appendix S3). However, individual application of potential management actions is often ineffective because it does not address all of the factors giving rise to the target barrier to restoration, does not address all barriers to restoration present in the site designated for restoration, and/or has unintended consequences. We believe that restoration outcomes can be improved by combining potential management actions into an integrated management effort (Figure 1.3).

Practicing restoration ecologists need to determine the type and extent of barriers to restoration that exist in the restoration site to inform the selection of management actions. If biological control organisms are available, classical biological control can be set up to reduce seed production and seed banks over time. If burning is appropriate, prescribed burning can be used to manage the litter. If burning is inappropriate, litter can be manually removed. Soil solarization or weed mats can be set up to reduce the establishment of secondary invaders, weedy native species, and/or a second generation of the target invader. Regardless of whether burning is applied, a combination of mowing, manual weeding, herbicides, or grazing can then be used to manage the resulting vegetation. Soil carbon addition and microbial treatments can be applied and then native species can be reintroduced, with or without the aid of nurse plants. Different combinations of approaches will be effective in different contexts (Figure 1.3). For example, a combination of prescribed burning, herbicide application, and native restoration species seeding improved restoration outcomes during restoration of prairies and oak savannas in the United States (Stanley et al., 2011). There is need for

further research on the efficacy of different integrated combinations of management approaches to address barriers to restoration presented by soil legacy effects of alien N₂-fixing woody species.

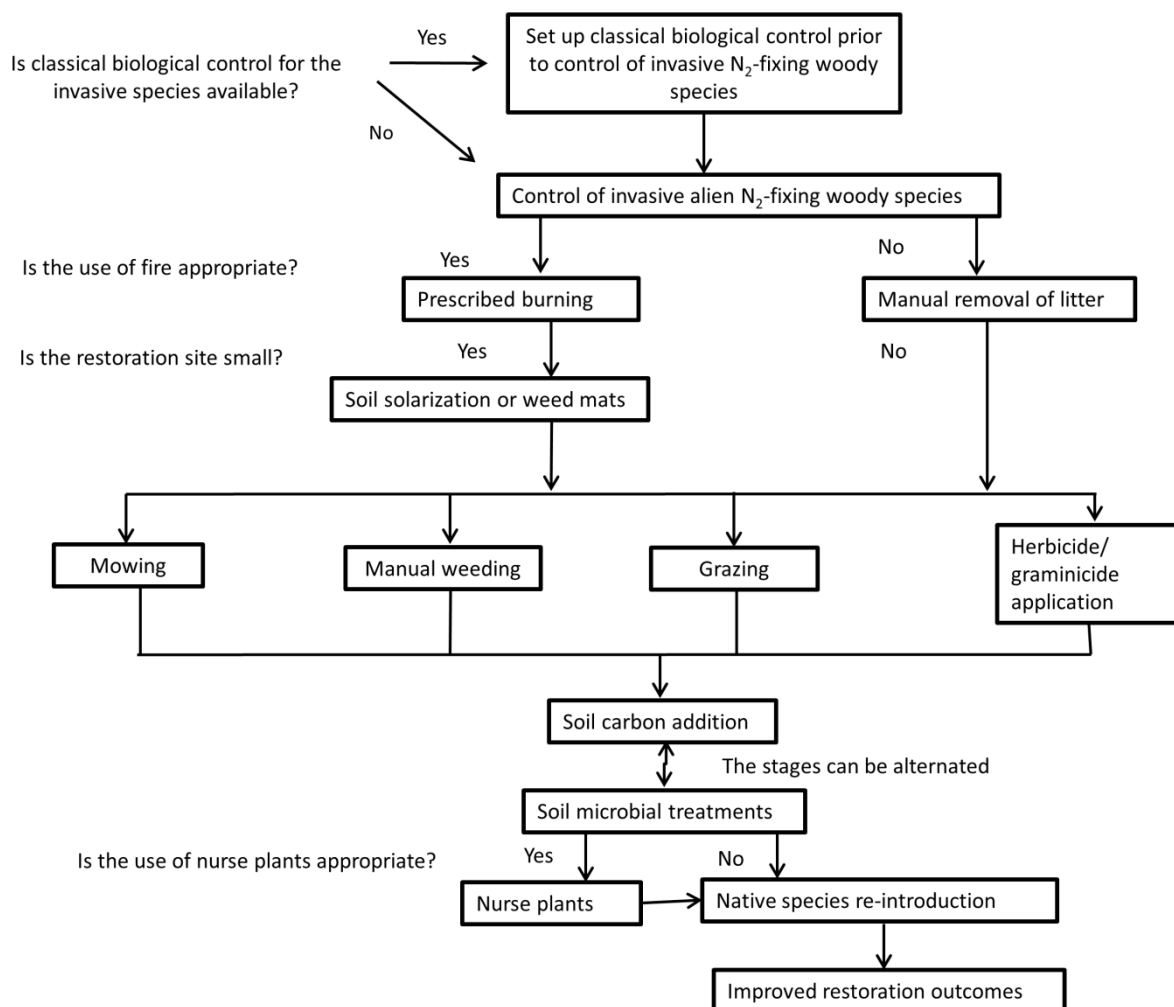


Figure 1.3: How to combine potential management actions to address the five barriers to restoration presented by soil legacy effects of invasive alien N₂-fixing woody species identified in this study into an integrated management effort to improve restoration outcomes.

1.8 Acknowledgments

Funding for this work was provided by the DST-NRF Centre of Excellence for Invasion Biology and Working for Water Program through their collaborative research project on “Integrated Management of invasive alien species in South Africa”. B.W.vW. thanks the National Research Foundation (grant 87550) for funding. We thank Mark Paschke and three anonymous reviewers for useful comments that helped to improve the manuscript.

Chapter two: *Acacia saligna*'s soil legacy effects persist up to 10 years after clearing: Implications for ecological restoration

2.1 Abstract

Management interventions such as control or eradication are usually necessary to reduce the negative impacts of invasive plants. It is often assumed that the impacts of invasive plants will diminish immediately after such interventions. However, in some cases the invader can have legacy effects in the soil that might persist for long periods, preventing the natural restoration of the areas managed. Therefore, to achieve the re-establishment of a functional native ecosystem it is important to understand for how long such legacies can persist in the soil. This paper explores this issue, using *Acacia saligna* in South Africa as case study. We collected soil samples in invaded, non-invaded and previously invaded sites (representing 2, 6 and 10 years after clearing) and analysed the levels of pH, carbon, nitrogen, available phosphorus, ammonium, nitrate and electrical conductivity. We also analysed enzyme activities (β -1,4-glucosidase, urease and acid phosphatase). *Acacia saligna* invasion alters overall soil characteristics but specifically raises pH by 0.6–1.8. Moreover, soil characteristics (e.g. pH) are not restored to natural conditions after control (soil legacy effects persist up to 10 years after clearing). Furthermore, *A. saligna* control elevates soil NO_3^- levels and these can remain higher than in invaded (1.55–6.67 mg kg^{-1}) and non-invaded (2.16–4.35 mg kg^{-1}) sites up to 10 years after clearing. Elevated NO_3^- often facilitates secondary invasion and/or weedy native species dominance which may hinder the restoration of functional native ecosystems. Therefore, strategies to manage areas previously invaded by *A. saligna* should take into account the removal of litter from the target invader, secondary invaders and weedy native species.

2.2 Introduction

Alien plant invasions are a global problem arising from the widespread translocation of plant species beyond their natural ranges to new areas where some of them become invasive (Richardson et al., 2011). Some invasive plants transform the ecosystems they invade (*sensu* Richardson et al., 2000; Ortega and Pearson, 2005) leading to negative impacts on native plant diversity, ecosystem function and ecosystem services (Vitousek et al., 1997; Pyšek and Richardson, 2010). Management interventions such as control or eradication are often necessary to reduce these negative ecological impacts (Pickart et al., 1998a; Malcolm et al., 2008; Moran and Hoffmann, 2012; Akamatsu et al., 2014).

It is often assumed that the negative impacts of invasive plant species will diminish immediately after such interventions (Wittenberg and Cock, 2005). However, this is not always the case. For example, the invader can leave legacy effects in the soil (e.g. altered soil chemical or physical characteristics, or altered soil microbial communities) that might persist after its removal thereby preventing the natural restoration of the area (*sensu* Corbin and D'Antonio, 2004; Marchante et al., 2009). In such cases, removal of the invader alone may not be sufficient to return the ecosystem to its original state (Macdonald, 2004; Marchante et al., 2009) – for example legacy effects in dune soils after the removal of the invader *Carpobrotus edulis* can facilitate the establishment of opportunistic species that out-compete native dune species, preventing their re-establishment (Novoa et al., 2013).

If soil legacy effects persist after removal of the invader, they can (i) facilitate re-invasion by the same invader or secondary invasion (Yelenik et al., 2004; Malcolm et al., 2008; González-Muñoz et al., 2012); (ii) prevent native plant species recovery and reestablishment (Maron and Jefferies, 1999) and (iii) be self-reinforcing and appear to promote an 'alternative stable state' which is difficult to successfully restore to pre-invasion conditions (Marchante et al., 2008; Suding et al., 2013; Gaertner et al., 2014).

Acacia saligna (Labill.) H.L Wendl. (Fabaceae) was introduced in various parts of the world for a wide range of commercial, subsistence and ornamental uses (Wilson et al., 2011). It is currently invasive in parts of Algeria, Chile, Cyprus, Israel, Italy, Kenya, Morocco, Portugal, South Africa and Spain (Thompson et al., 2015), covering an estimated area of 600 000 ha worldwide (Griffin et al., 2011). In South Africa, *A. saligna* covers approximately 53 000 ha (van Wilgen et al., 2011). In the invaded ecosystems, *A. saligna* alters soil characteristics – mainly through nitrogen fixation, litter production (Musil and Midgley, 1990; Witkowski, 1991a; Musil, 1993; Yelenik et al., 2004) and changes in soil microbial community structure, diversity and function (Crisóstomo et al., 2013). In fact, the invasion of *A. saligna* in South Africa has resulted in the development of extensive woodlands dominated by the alien species and a corresponding decrease in native plant diversity (Le Maitre et al., 2011; van Wilgen et al., 2011; Gaertner et al., 2012a).

There are numerous efforts to globally control *A. saligna* invasions (Le Maitre et al., 2011) and some of them are successful in reducing its distribution (van Wilgen et al., 2011). For example, a fire of moderate intensity is often applied after clearing acacias to (i) consume the litter – *A. saligna*'s litter can supplement soil nitrogen and/or form a physical barrier to

re-establishing native seedlings (Richardson and Kluge, 2008), and (ii) reduce the amount of *A. saligna*'s seeds in the seed bank – by destroying the seeds or triggering mass germination (Milton and Hall, 1981). However, most efforts to restore controlled areas fail because the re-introduced native plant species do not establish (Gaertner et al., 2012a). This might be due to secondary invasion – that is an increase in the abundance of non-target alien plant species that establish after clearing the target invader (Pearson et al., 2016), and/or weedy native species dominance – that is an increase in the abundance of native species that are not typically distributed in the area and are often found dominating disturbed environments (chapter three). Furthermore, the failure of such restoration efforts has been associated with strong inhibition caused by soil legacy effects (Macdonald, 2004; Gaertner et al., 2012b). However, limited attention has been given to understanding the soil legacy effects of *A. saligna* (or invasive Australian acacias in general) after clearing (see Yelenik et al., 2004; Marchante et al., 2009). This paper explores the issue of persistent soil legacy effects, using invasive *A. saligna* in South Africa as case study. Overall, we set out to (i) determine how soil characteristics differ between cleared, invaded and non-invaded sites with emphasis on time after clearing, and (ii) understand the implications that the differences in soils can have for ecological restoration.

2.3 Materials and Methods

2.3.1 Study sites

The study was conducted in the Cape Flats Sand Fynbos (CFSF) of the Western Cape Province, South Africa – a main hotspot for *A. saligna* invasion in the South African fynbos. The climate in the CFSF is Mediterranean with hot dry summers and cool wet winters, and the soils are broadly classified as well-drained aeolian acidic sands characterized by low nitrogen (Rebelo et al., 2006; Musil, 1993). Native CFSF vegetation consists of evergreen shrub-lands dominated by a mixture of ericoid and proteoid shrubs and restioid (aphyllous graminoid) growth forms (Rebelo et al., 2006). Cape Flats Sand Fynbos is a critically endangered vegetation type with less than 1% of the total historical area statutorily conserved and it is the most transformed of the sand fynbos types occurring in the Greater Cape Floristic Region (Rebelo et al., 2006).

We selected three study sites in the CFSF, namely, Blaauwberg Nature Reserve (33°4605.16"S; 18°27010.08"E), Youngsfield (34°0030.30"S; 18°29016.20"E) and Penhill (33°5900.39"S; 18°43037.74"E). Youngsfield and Penhill had similar soil types, whereas

Blaauwberg Nature Reserve differed slightly (Table 2.1). The three study sites received different amounts of annual precipitation (i.e. between 361 and 1018 mm; Table 2.1) which can affect some soil characteristics such as electrical conductivity (Rhoades et al., 1976; Sudduth et al., 2005).

In each study site, we delimited three areas: non-invaded, densely invaded and cleared of invasive *A. saligna* and left to recover naturally. Invaded areas were densely invaded (75–100% cover) and had been invaded for a ‘long time’ (>20 years; see Marchante et al. (2008) for definition/classification). Each site had experienced at least one fire in the last 10 years, with the time since the last fire and soil sampling for this study ranging from 4 to 7 years (Table 2.1). The time between clearing and sampling was between 2 and 10 years, and the cleared areas received between one and seven follow-up treatments, in which re-emerging invader seedlings were removed through weeding or herbicides (Table 2.1). Only one cleared area (Penhill) was burnt immediately after clearing. Cleared areas were dominated by secondary invaders such as *Briza maxima* L., and weedy native species such as *Ehrharta calycina* Sm. (chapter three; chapter four). Non-invaded areas were characterized by mature native fynbos plant communities, were free from *A. saligna* invasion and we believe that they represent the environmental characteristics of densely invaded sites prior to invasion. Non-invaded areas were kept free from *A. saligna* invasion through management and not as a result of different environmental characteristics. The study sites varied in size from 1.5 to 9.5 km².

2.3.2 Soil collection

Soil samples were collected in the cleared, invaded and non-invaded area of each study site. In each cleared, invaded or non-invaded area, we chose five random sampling points (0.5 x 5 m; $n = 45$). In June 2015, five soil sub-samples (approximately 600 g each) were taken from the top 10 cm at each sampling point after removing the organic horizon material. All five sub-samples per sampling point were bulked and homogenized to make a representative sample.

Table 2.1: History of study sites including the mean annual precipitation; soil type; number of years after initial clearing; number of follow-up *A. saligna* clearing treatments; years since last fire and whether the cleared site was burnt after initial *A. saligna* clearing.

Study site	Mean annual precipitation (mm)	Years after initial clearing	Years since fire	Post clearing burn?	Number of follow-up treatments	Soil type (http://bgisviewer.sanbi.org)
Blaauwberg Nature Reserve	361	2	4	No	1	Greyish sandy soil
Youngsfield	1018	6	6	No	4	Soils with a sandy texture, leached and with sub-surface accumulation of organic matter and aluminium with/without oxides, either deep or on hard or weathering rock
Penhill	556	10	7	Yes	7	Soils with a sandy texture, leached and with sub-surface accumulation of organic matter and aluminium with/without oxides, either deep or on hard or weathering rock

2.3.3 Soil analysis

Approximately 500 g of soil per sample were taken to Bemlab Pty Ltd (Somerset West, Western Cape) and analysed for pH, % carbon (C), % nitrogen (N), available phosphorus (Bray II; AP), ammonium (NH_4^+), nitrate (NO_3^-) and electrical conductivity (EC). PH (1.0 M KCl), EC and AP were analysed according to the methods given by the Non-affiliated Soil Analysis Work Committee (1990). C and N were determined through total combustion using a Leco Truspec CHN analyser. NH_4^+ and NO_3^- were extracted from soil with 1 N KCl and determined colorimetrically on a SEAL AutoAnalyzer 3 (*sensu* AgriLASA, 2004).

2.3.4 Enzyme analysis

Soil microbial communities release extracellular enzymes that allow them to access energy and nutrients present in complex substrates and initiate the processes of decomposition and nutrient mineralization (Allison and Vitousek, 2005). Enzyme activities can be used to understand the key processes linking soil microbial populations and nutrient dynamics (Sinsabaugh and Moorhead, 1994), and understanding the effects of plant invasions (e.g. Vilà et al., 2011; Novoa et al., 2013; Souza-Alonso et al., 2015). In this study, we analysed the activities of the enzymes β -1,4-glucosidase (E.C. 3.2.1.21; glucosidase); urease (E.C. 3.5.1.5); and acid phosphatase (E.C. 3.1.3.2; phosphatase) responsible for the cycles of C, N and P respectively.

Approximately 100 g of soil per sample were taken to the molecular ecology lab (Department of Botany and Zoology, Stellenbosch University) in October 2015 and analysed for enzyme activities. The substrates used for the glucosidase, urease and phosphatase assays were *p*-nitrophenol (*p*NP)- β -D glucopyranoside, urea and *p*NP-phosphate respectively. The Modified Universal Buffer (20.14 mmol L⁻¹ Tris-hydroxymethyl aminomethane; 17.3 mmol L⁻¹ Malic acid; 14.6 mmol L⁻¹ Citric acid; 20.3 mmol L⁻¹ Boric acid and 1 mol L⁻¹ NaOH) was used to make the substrates. There were six analytical replicates and six sample controls of each treatment. For the analysis of glucosidase, urease and phosphatase, the methods followed were those described by Novoa et al. (2014).

2.3.5 Statistical analyses

The data were analysed using R version 3.3.0 (R Development Core Team, 2016). All soil characteristics and enzyme activities were compared separately – that is only within each study site to avoid unexplainable variation in soils resulting from differences in soil type and

micro-climates between study areas. For testing the effect of invasion status (invaded, cleared and non-invaded) on overall soil nutrient composition in each study site, we conducted a principal components analysis (PCA) on the correlation matrix of the soil nutrient variables using the *base* package. After inspection of PCA plots, we conducted a Permutation Multivariate Analysis of Variance (PERMANOVA; Anderson, 2001) with 9999 permutations using Euclidean distance (to correspond to the PCA distance) with the function *adonis* from the *vegan* package (Oksanen et al., 2014) as an additional test. We first standardized all the variables to mean zero and unit variance and then included invasion status as predictor variable. For testing the effect of invasion status on the individual soil nutrient levels and enzyme activities in each study site, we conducted a one-way Analysis of Variance (ANOVA). Available phosphorus and ammonium were log transformed to accomplish the normality assumption of ANOVA. Significant mean differences were separated with Tukey's HSD test using *multcomp* package (Hothorn et al., 2008).

2.4 Results

2.4.1 Overall soil characteristics between invaded, cleared and non-invaded areas

The PCAs conducted per study site were based on five replicates per invasion status and they separated the three invasion statuses (Figure 2.1). For Blaauwberg Nature Reserve, the first two principal components explained 67% of variation (PC1 = 45%; PC2 = 22%) in the dataset. PC1 separated the cleared from the invaded and non-invaded areas, whereas PC2 separated the non-invaded from the invaded and cleared areas. Variables that contributed the most to PC1 were NO_3^- , AP and EC, and for PC2 were pH and NH_4^+ . For Youngsfield, the first two principal components explained 63% of the variation (PC1 = 36%; PC2 = 27%) in the dataset. The invaded, cleared and non-invaded areas were different to each other in terms of PC1 and PC2. Variables that contributed the most to PC1 were N, NO_3^- and AP, and for PC2 were C and pH. For Penhill, the first two principal components explained 62% of the variation (PC1 = 39%; PC2 = 23%) in the dataset. PC1 separated the three areas from each other. Variables that contributed the most to PC1 were NO_3^- , pH and AP, and for PC2 were C and NH_4^+ . PERMANOVA results showed that the overall soil characteristics between all cleared sites and their reference invaded and non-invaded sites were significantly different (Table 2.2).

Table 2.2: PERMANOVA results for each study area based on soil nutrient levels and invasion status of sites within the areas. Significance indicated in bold as: * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$.

Site name	Factor	Df	Sums Of Squares	Mean Squares	F. Model	R ²	p
Blaauwberg Nature Reserve	Invasion status	2	34.676	17.338	3.2856	0.35384	0.0019**
	Residuals	12	63.324	5.277	0.64616		
	Total	14	98	1			
Youngsfield	Invasion status	2	54.441	27.22	7.4988	0.55552	<0.001***
	Residuals	12	43.559	3.63	0.44448		
	Total	14	98	1			
Penhill	Invasion status	2	42.854	21.427	4.6626	0.43728	<0.001***
	Residuals	12	55.146	4.5955	0.56272		
	Total	14	98	1			

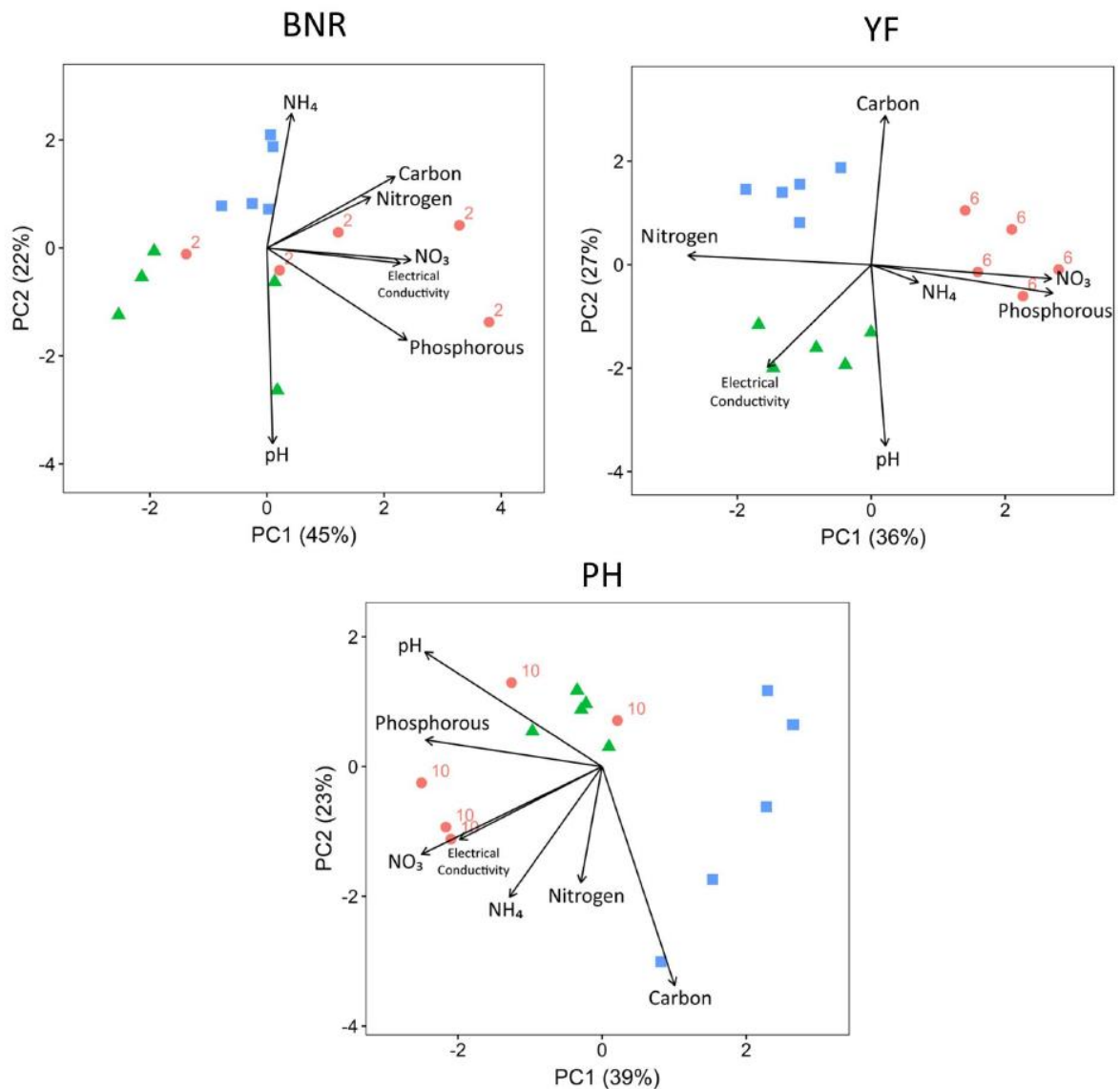


Figure 2.1: Overall soil nutrient composition in the different *A. saligna* invasion statuses in each study area. Dots = cleared sites; triangles = invaded sites; and squares = non-invaded sites. BNR = Blaauwberg Nature Reserve; PH = Penhill; and YF = Youngsfield.

2.4.2 Individual soil nutrient levels and enzyme activities, pH and EC between cleared, invaded and non-invaded sites

We believe that prior to invasion, each densely invaded area had similar soil characteristics to its reference non-invaded area. Of the soil chemistry characteristics investigated, invasion significantly elevated pH levels by 0.6–1.8, and lowered C content by 75% at Youngsfield and Penhill (Figure 2.2). The remaining characteristics were unaffected. Clearing of invaded sites elevated NO₃⁻ levels and these remained higher than the invaded (1.55–6.67 mg kg⁻¹) and non-invaded (2.16–4.35 mg kg⁻¹) areas up to 10 years after clearing

(Figure 2.2). In addition, the cleared area (6 years after clearing) at Youngsfield, had lower N and EC levels (88% and 49% respectively) but higher (252%) AP. The cleared area (10 years after clearing) at Penhill also had higher (136%) AP. After clearing, NO_3^- (2.16–4.35 mg kg^{-1}) and pH (0.88–1.76) levels remained higher up to 10 years after clearing (Figure 2.2). Furthermore, the cleared areas at Youngsfield and Penhill had higher levels of AP, NO_3^- and pH, whereas the cleared area at Youngsfield had lower (89%) N content. There were no significant differences in the activity of glucosidase, phosphatase and urease among all cleared sites and their reference invaded and non-invaded sites (Figure 2.3).

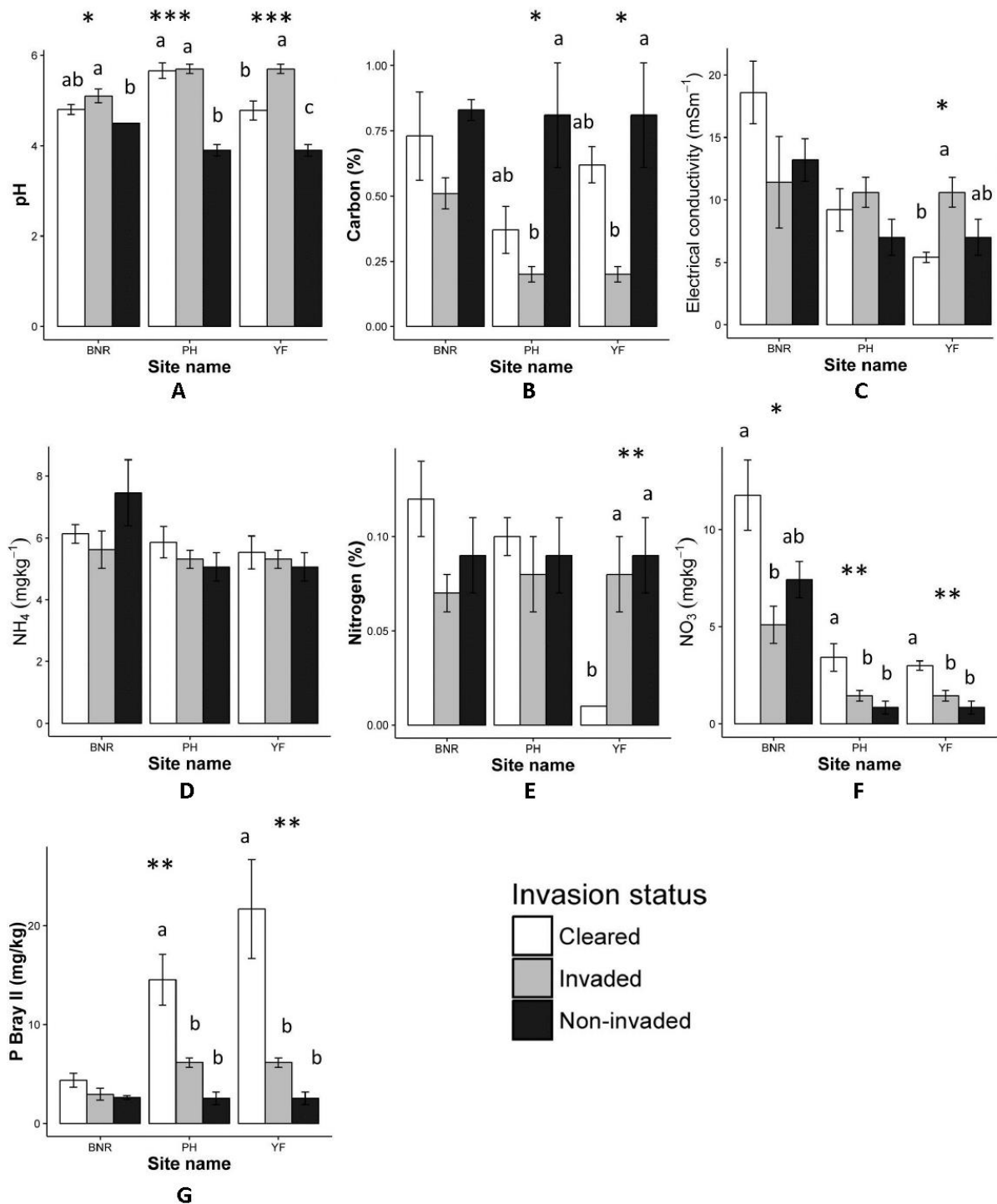


Figure 2.2: Levels of pH (A), carbon (B), electrical conductivity (C), NH₄⁺ (D), nitrogen (E), NO₃⁻ (F) and available phosphorus (G) in the different *A. saligna* invasion statuses in each study area. Mean values of each soil nutrient in each study area with the same letter are not significantly different. Significance indicated in bold as: * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$. BNR = Blaauwberg Nature Reserve; PH = Penhill; and YF = Youngsfield.

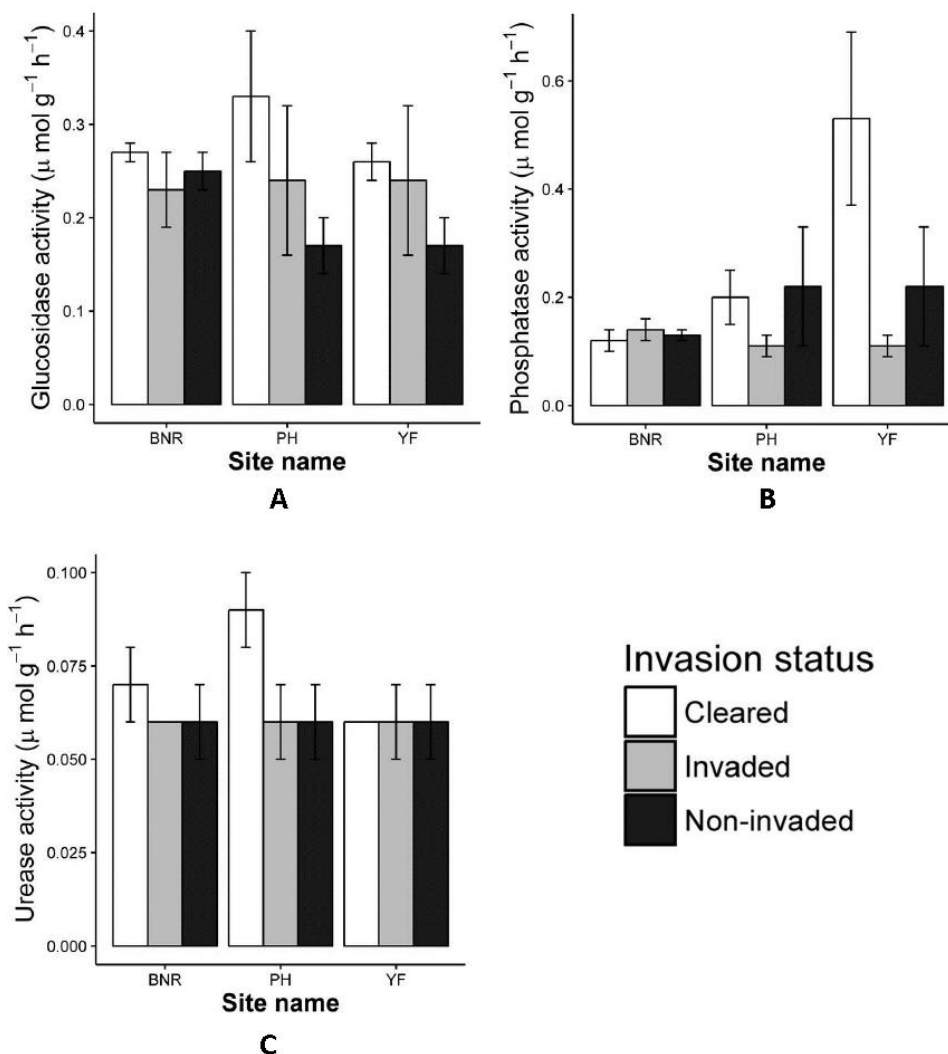


Figure 2.3: Glucosidase (A), phosphatase (B) and urease (C) activity in the different *A. saligna* invasion statuses in each study area. BNR = Blaauwberg Nature Reserve; PH = Penhill; and YF = Youngsfield.

2.5 Discussion

2.5.1 Impacts of *Acacia saligna* on soil characteristics

Our results showed significant differences in pH and carbon levels between non-invaded and invaded areas. These observations support previous studies which showed that a significant proportion of invasive alien species, particularly N_2 -fixing species, alter soil characteristics and processes in the areas they invade (Vitousek, 1990; Stock et al., 1995; Gordon, 1998; Maron and Jeffries, 2001; Marchante et al., 2008; Von Holle et al., 2013).

First, our results suggest that invasion by *A. saligna* increases soil pH levels (makes them more basic). Native South African fynbos is characterized by acidic soils (Rebelo et al.,

2006). The decomposition of the large amounts of litter that *A. saligna* adds to the invaded soils might cause the observed increase on such low pH levels (Ehrenfeld, 2003, 2004; Raizada et al., 2008). Various mechanisms might explain why litter decomposition can increase the soil pH – for example release of NH_3 from the decomposing litter or depletion of protons in the soil through oxidation of organic anions (Noble et al., 1996). Increases in pH have been generally observed with both N_2 -fixing (e.g. *A. saligna* in the South African fynbos; Gaertner et al., 2011; Mostert et al., 2016) and non- N_2 -fixing alien plant invaders (e.g. *Berberis thunbergii* and *Microstegium vimineum* in New Jersey (USA); *Parthenium hysterophorus* in grassland communities of central Nepal; Timsina et al., 2011). Second, C content was lower in the invaded areas at Youngsfield and Penhill in comparison to non-invaded areas. This is probably a result of previous fire events in the study sites (Table 2.1) that most likely burnt off the soil organic material that was otherwise high in C content. Soil C content was also reported lower in Portuguese coastal dunes invaded by *Acacia longifolia* that experienced fire compared to non-invaded areas that had not been burnt (Marchante et al., 2008). Generally, soil C content may decrease after one or more fire events (Carreira et al., 1994; Paul et al., 2002) and the intensity of the fire can affect the amount of C lost (Paul et al., 2002).

However, we found no differences in N or AP between invaded and non-invaded areas. Previous studies have shown elevated N levels associated with the invasion of N_2 -fixing plants (Vitousek and Walker, 1989; Stock et al., 1995; Pickart et al., 1998a; Malcolm et al., 2008; Marchante et al., 2008; González-Muñoz et al., 2012; Grove et al., 2015). The reason behind this might be that the reference non-invaded areas of our study showed higher N levels than expected. We also observed non-significant differences in nitrogen and phosphorus availability (i.e. NO_3^- ; NH_4^+ ; AP) between invaded and non-invaded areas. These observations support previous findings on *A. saligna* and *Acacia cyclops* invasions (Stock et al., 1995; Gaertner et al., 2011). The magnitude of the effect of *Acacia* invasions on nitrogen or phosphorus availability may differ depending on the leaf chemistry of the *Acacia* spp. and/or the soil properties of the study site (Stock et al., 1995).

2.5.2 Legacy effects of *Acacia saligna* on soil characteristics

Numerous studies have observed persistence of altered soil characteristics for long periods after clearing invasive alien N_2 -fixing species – for example more than 4 years in the pine-oak systems (USA) cleared of *Robinia pseudoacacia* (Malcolm et al., 2008) and

Portuguese coastal dunes cleared of *A. longifolia* (Marchante et al., 2009). Our results show that pH levels can either be recovered after *A. saligna* is controlled – that is in the cleared sites at Blaauwberg Nature Reserve and Youngsfield, pH levels were intermediate to the invaded and non-invaded sites – but they can also remain similar to invaded sites – that is in the cleared site at Penhill they were similar to the invaded site. These observations suggest that the impact of *A. saligna* on pH can be profound and persist up to 10 years after clearing. Altered pH levels often exert negative impacts on fungal and bacterial compositions (Bååth and Anderson, 2003), enzyme activities (Dick et al., 1988) and availability of some nutrients such as manganese and zinc (Sims, 1986).

Moreover, 6 years after clearing, we found a low N compared to the reference invaded and non-invaded areas. The differences are most likely a result of high N losses in the cleared area (Stock and Lewis, 1986; Witkowski, 1991a; Malcolm et al., 2008; Jovanovic et al., 2009; Staska et al., 2014). It is worth noting that low N in the cleared area compared to the invaded and non-invaded areas were not standard across all study sites. All our study sites were located on coarse-textured soils which are known to have a lower water holding capacity and a greater potential to lose N from organic matter and NO_3^- through leaching (Stock and Lewis, 1986; Witkowski, 1991a). Therefore, we suspect that the lower water holding capacity in combination with high precipitation and uptake by secondary invaders and weedy native species (personal observation) in the cleared area resulted in higher N losses compared to the reference invaded and non-invaded areas.

Our results suggest that clearing invasive *A. saligna* increases NO_3^- levels, and these changes persist up to 10 years after clearing. Such changes might be a result of a stimulation of the nitrification process with clearing – that is the initial effect of clearing the invader allows the sun to reach the ground previously covered by the invader canopy, which increases temperatures in the soil stimulating the nitrification process (Yelenik et al., 2004). We suspect that with time (2, 6 and 10 years after clearing) some of the NO_3^- may be lost through leaching and taken up by secondary invaders and/or weedy native species (Stock and Lewis, 1986; Jovanovic et al., 2009; Gaertner et al., 2011; Staska et al., 2014). However, we believe that NO_3^- levels are often high after initial clearing such that the losses may not be high enough to have an overall effect. We also suspect that decomposition of organic matter from *A. saligna* cleared and left on site after follow-up treatments, secondary invaders and/or weedy native species in these sites (personal observation) may contribute to maintaining elevated NO_3^- levels in the cleared sites (Symstad, 2004; Chapman et al., 2006).

Phosphorus availability was higher 6 and 10 years after clearing in comparison to the reference invaded and non-invaded areas. This might be a result of the decomposition of organic matter from *A. saligna* cleared and left on site after follow-up treatments, secondary invaders and/or weedy native species in these sites (personal observation). Organic matter can elevate available phosphorus in two ways (i) decomposition of organic matter can release phosphorus products that can be available in the soil solution (Guppy et al., 2005), and/or (ii) competition between the decomposition products of organic matter and P for soil sorption sites can result in phosphorus being more available in the soil solution (Guppy et al., 2005). Phosphorus is a limiting nutrient in fynbos soils (Groves, 1983). Therefore, depending on the species and type of fynbos, elevated AP after clearing can restrict fynbos species distribution, favour the dominance of secondary invaders and weedy native species (Power et al., 2010).

Finally, the C content in the sites 6 and 10 years after clearing was intermediate to the reference invaded and non-invaded areas suggesting that the C content of the soil was on the path to recovery from the effect of fire events experienced in the sites, most likely with the aid of accumulating secondary invader and/or weedy native species litter (Dumontet et al., 1996).

2.5.3 *Soil enzyme activities*

Extensive evidence from previous studies has shown that soil microbiota is highly responsive to the type of plant species occupying the soil; hence changes in species composition and pH that come with invasion are expected to alter the structure and/or functional properties of soil microbiota (Ehrenfeld, 2004; Fierer and Jackson, 2006; Lauber et al., 2008; Vilà et al., 2011). Numerous studies of *A. saligna* invasion have suggested that the species produces large amounts of litter with high N content resulting in more N returning from the above-ground biomass to the soil (Witkowski, 1991a; Stock et al., 1995; Yelenik et al., 2004).

In light of current knowledge, we expected differences in the activity of enzymes related to N and C cycling (urease and glucosidase), and P cycling (phosphatase) between invaded, cleared and non-invaded areas; however, we did not observe this. Instead our results conflict with numerous studies that have shown a significant increase in enzyme activity under the invader canopy compared to native stands (Kourtev et al., 2002; Allison et al., 2006; Caldwell, 2006; Marchante et al., 2008; Vilà et al. 2011). We suspect that enzyme activities in our study were limited by moisture availability (Yelenik et al., 2004) which has been

shown to limit the decomposition process in the South African fynbos around the time we sampled for soil enzyme activity (summer; Witkowski, 1991a). Low moisture content could have resulted from the hot and dry conditions in our study areas (Rebelo et al., 2006) and compounded by rapid surface drying caused by the significant percentage of coarse sand in the soils (Witkowski, 1991a; M.M. Nsikani, unpublished data). Low soil moisture can strongly limit enzyme activities in the soil (Steinweg et al., 2012). It may be advisable for future studies to sample during the hot and dry summer, and the cold and wet winter, to accurately sample for soil enzyme activities in the South African fynbos.

2.6 Conclusions and implications for restoration

Overall, our findings suggest that invasion of native areas by the alien *A. saligna* (i) changes overall soil characteristics; and (ii) leaves soil legacies that can persist up to 10 years after clearing. Therefore, clearing the invader does not reverse these effects; instead it elevates soil NO_3^- levels. Elevated NO_3^- levels after clearing *A. saligna* often facilitate secondary invaders (Yelenik et al., 2004; Pearson et al., 2016) and weedy native species that often out-compete native species (Yelenik et al., 2004). We acknowledge that single sampling can sometimes be misleading and we urge future studies to sample more than once per year at different seasons.

To our knowledge, there is no known management strategy that can be successfully used to facilitate the recovery of overall soil characteristics and elevated NO_3^- levels after clearing invasive *A. saligna*. However, we believe that active restoration by planting native species could, over time, return soils to previous conditions. Many species adapted to low N availability (e.g. typical native fynbos species) can (i) immobilize N and slow N cycling using various mechanisms, such as producing relatively recalcitrant and high C:N litter; and (ii) restore ecosystem function, when they successfully re-establish (Perry et al., 2010). However, re-establishment of such planted native species after clearing *A. saligna* could be elusive due to competition with secondary invaders and/or weedy native species (Yelenik et al., 2004; chapter three; chapter four). Strategies to reduce soil N availability and their efficacy have been reviewed by Perry et al. (2010). We urge practising restoration ecologists to consider them when managing sites previously invaded by *A. saligna* to facilitate the recovery of elevated NO_3^- levels. Moreover, we believe that litter from the target invader, secondary invaders and weedy native species should be removed to aid the recovery of pH and available phosphorus after clearing. Manual removal of invader litter layer using a rake

or similar instrument (small invader populations; Pickart et al., 1998a) or fire (extensive invasions; Richardson and Kluge, 2008) can be used to remove the invader litter layer.

We believe that restoration efforts after clearing invasive *A. saligna* should not focus on restoring native soil characteristics because (i) there are very few management strategies that can be implemented and success is not guaranteed, and (ii) altered soil characteristics per se often do not have negative effects on the re-establishment of native species (chapter three). Instead restoration efforts need to focus on conducting multiple successive follow-up treatments to avoid re-invasion by *A. saligna* (Van Wilgen et al., 2011), and the removal of secondary invaders and weedy native species (Pearson et al., 2016).

2.7 Acknowledgements

Funding for this work was provided by the DST-NRF Centre of Excellence for Invasion Biology and Working for Water Program through their collaborative research project on “Integrated Management of invasive alien species in South Africa”. BWvW thanks the National Research Foundation (grant 87550) for funding. AN was supported by project no. 14-36079G Centre of Excellence PLADIAS (Czech Science Foundation) and long-term research development project RVO 67985939 (The Czech Academy of Sciences). We sincerely thank the City of Cape Town for giving us permission to use their sites for data collection and Johannes J. Le Roux for his assistance in designing the study.

Chapter three: Re-establishment of *Protea repens* after clearing invasive *Acacia saligna*: Consequences of soil legacy effects and a native nitrophilic weedy species

Please use the link below to access the full list of appendices:

<https://doi.org/10.1016/j.sajb.2018.02.396>

3.1 Abstract

Invasive Australian acacias can alter soil chemistry and microbial communities in areas they invade. After clearing invasive acacias, these changes can persist, and previously invaded areas can become dominated by nitrophilic weedy species. Restoration of viable native plant communities in cleared sites often fails due to a lack of native species re-establishment. Therefore, to improve restoration outcomes, it is important to understand the effects of soil chemical and biotic legacies, and of nitrophilic weedy species, on native species re-establishment. We germinated and grew *Protea repens* seedlings (a native proteoid shrub) as an indicator species in soil taken from areas cleared of *Acacia saligna* in lowland fynbos, as well as from non-invaded areas under controlled conditions, to investigate the effect of soil chemical legacies. We sterilized half the soil from each cleared or non-invaded area to investigate the effect of soil biotic legacies. We grew *Ehrharta calycina* (a native nitrophilic weedy grass species) in half of each treatment and measured the effect of treatments on *P. repens* germination and growth. Germination percentage, root and shoot dry mass of *P. repens* did not significantly differ between altered and native soil chemistry. The germination percentage of *P. repens* was significantly greater (93%) in the presence of soil microbial communities than in their absence. The presence of *E. calycina* significantly increased (29%) the root-to-shoot ratio of *P. repens* than in their absence. Since the legacy of altered soil chemistry did not have a direct negative effect on *P. repens* germination and growth; we conclude that restoration efforts do not always have to manage altered soil chemistry after clearing invasive *A. saligna*.

3.2 Introduction

Australian acacias have been introduced to many parts of the world for various purposes (Richardson et al., 2011). A majority of these introductions have resulted in naturalization and ultimately widespread invasion (Richardson and Rejmánek, 2011). Most of the areas invaded by Australian acacias (e.g. South African lowland fynbos and Portuguese dune systems) have nutrient-poor soils characterized by low levels of nitrogen (Musil, 1993;

Marchante et al., 2009). Invasive Australian acacias are ‘ecosystem transformers’ as they can alter ecosystem processes and functions (*sensu* Richardson et al., 2000; Ehrenfeld, 2003). Invasion by acacias can alter the soil chemistry through mechanisms such as nitrogen fixation and litter production (Witkowski, 1991b; Musil, 1993; Yelenik et al., 2004; Marchante et al., 2008); change the soil microbial community composition and function using mechanisms such as introduction of novel microbes and deposition of allelochemicals (Inderjit and van der Putten, 2010; Crisóstomo et al., 2013; Rodríguez-Echeverría et al., 2013; Lorenzo et al., 2013), exclude native species through competition (Gaertner et al., 2012a; González-Muñoz et al., 2012), and create feedback loops that favour their dominance (Gaertner et al., 2014).

It is often assumed that the negative impacts of invasive species will diminish after clearing the invasive species (Wittenberg and Cock, 2005). However, the negative impacts of invasive acacias can persist for long periods despite clearing the invasive species – i.e. they become legacy effects (Marchante et al., 2009; Rodríguez-Echeverría et al., 2013; Lazzaro et al., 2014; Souza-Alonso et al., 2014; chapter two). Legacy effects include measurable changes in biological, soil chemical or physical conditions that persist after clearing the invasive species (*sensu* Corbin and D’Antonio, 2004).

Efforts to restore functional native plant communities are underway after clearing invasive acacias (Marchante et al., 2009; Le Maitre et al., 2011; Gaertner et al., 2012a). Restoration measures often include removing the nitrogen-rich invader litter left over after clearing (Marchante et al., 2009; Le Maitre et al., 2011), removing *Acacia* seedlings that germinate following clearing of adult plants (Marchante et al., 2010; Krupek et al., 2016) and re-introducing native species (Marchante et al., 2004; Gaertner et al., 2012a, 2012b). However, it has proved difficult to successfully restore functional native plant communities in cleared areas due to a lack of native species re-establishment (Galatowitsch and Richardson, 2005; Marchante et al., 2011). The lack of native species re-establishment is often associated with depleted native soil seed banks (Holmes and Cowling, 1997a; Le Maitre et al., 2011; González-Muñoz et al., 2012).

The legacy of altered soil chemistry after clearing invasive acacias such as elevated nitrogen availability has been shown to persist for up to ten years (Marchante et al., 2009; chapter two). Several studies have suggested that this legacy effect might directly have negative consequences for native species re-establishment by affecting their germination, growth, and/or indirectly by giving a competitive advantage to nitrophilic weedy species

(Marchante et al., 2008; Marchante et al., 2009; Le Maitre et al., 2011). Several vegetation surveys have reported that alien and/or native nitrophilic weedy species often dominate areas cleared of invasive acacias (Galatowitsch and Richardson, 2005; Blanchard and Holmes, 2008; Gaertner et al., 2012b; Fill et al., 2018). Soil microbial communities can influence the germination and growth of species during restoration (Balshor et al., 2017). Several studies have suggested that the legacy of altered soil microbial communities can have a negative influence on native species re-establishment through different mechanisms such as accumulation of pathogenic microbes and/or disruption of beneficial mutualisms (Bever et al., 1997; Eppinga et al., 2006; Callaway et al., 2008; Grove et al., 2017).

Currently there is limited knowledge on the effect of soil chemical and biotic legacies and nitrophilic weedy species after clearing invasive acacias on the re-establishment of proteoid shrubs. Furthermore, after clearing invasive acacias, previous studies have often used field observations instead of controlled experiments to reach conclusions about the effect of soil legacies and nitrophilic weedy species on the re-establishment of proteoid shrubs (e.g. Gaertner et al., 2012b; Fill et al., 2018). However, native species re-establishment under field conditions is affected by multiple interacting factors such as water availability and light. Using *Acacia saligna* (Labill.) H. L Wendl. (Fabaceae) invasions in the South African lowland fynbos as case study, we conducted a greenhouse experiment to investigate how germination and growth of a native proteoid shrub is affected by the invasive species' (i) soil chemical legacy, (ii) soil biotic legacy, and (iii) a native nitrophilic weedy species.

3.3 Materials and Methods

3.3.1 Study sites

The study was conducted using soils collected from the Cape Flats Sand Fynbos (CFSF) of the Western Cape Province in South Africa, where invasion by *A. saligna* is common. The climate in the CFSF is Mediterranean with cool wet winters and hot dry summers and the soils are broadly classified as well-drained aeolian acidic sands (Rebelo et al., 2006; Musil, 1993). Native CFSF vegetation consists of evergreen shrublands dominated by a mixture of ericoid and proteoid shrubs, and restioid (aphyllous graminoid) growth forms (Rebelo et al., 2006). Cape Flats Sand Fynbos is a critically endangered vegetation type with approximately 2% of the total historical area statutorily conserved and it is the most transformed of the sand fynbos types occurring in the Greater Cape Floristic Region (Rebelo et al., 2006). We

selected three study sites in the CFSF, namely, Blaauwberg Nature Reserve (33°4605.16"S; 18°27010.08"E), Youngsfield (34°0030.30"S; 18°29016.20"E) and Penhill (33°5900.39"S; 18°43037.74"E). The study sites varied in size from 1.5 to 9.5 km². Youngsfield and Penhill had similar soil types, whereas Blaauwberg Nature Reserve differed slightly (Table 2.1). Mean annual precipitation was between 361 and 1018 mm at the three study sites (Table 2.1).

In each study site, we selected non-invaded areas and previously invaded areas that had been cleared of invasive *A. saligna*. Each cleared area had experienced at least one fire in the last ten years (Table 2.1). The time between clearing and soil collection was between two and ten years, and the cleared areas received between one and seven follow-up treatments, in which re-emerging *A. saligna* seedlings were removed through weeding or herbicides (Table 2.1). Only one cleared area (Penhill) was immediately burnt after clearing. Cleared areas were dominated by nitrophilic weedy species such as *Briza maxima* L. and *Ehrharta calycina* Sm. (chapter three; chapter four). Non-invaded areas were characterized by mature native fynbos plant communities and were free from *A. saligna* invasion. We believe that the non-invaded areas closely represent the environmental characteristics of cleared areas prior to invasion and clearing. Non-invaded areas were kept free from *A. saligna* invasion through management and not as a result of different environmental characteristics. Each cleared area was characterised by different soil chemistry from its reference non-invaded area (chapter two).

3.3.2 Study species

The native shrubs of the Proteaceae family are amongst the species that are most affected by *A. saligna* invasion in the CFSF (Holmes and Cowling, 1997a). Serotinous Proteaceae are easily lost following dense invasions because they have no persistent seed banks once the adults have died (Holmes and Cowling, 1997a). Therefore, they are a prime target for restoration. *Protea repens* was historically widespread in the CFSF but its range has been severely reduced by invasive acacias (Witkowski, 1991b). Therefore, we chose *P. repens* as a representative of proteoid shrubs for purposes of this study. *Ehrharta calycina* (Sm.)(Poaceae) is a native re-sprouting perennial grass that often dominates sites cleared of invasive acacias and disturbed environments (Yelenik et al., 2004; Fill et al., 2018). Therefore, we chose it as a representative of nitrophilic weedy species for purposes of this study. Seeds of *P. repens* and *E. calycina* harvested from populations of wild plants were obtained from Vula Environmental Services.

3.3.3 Study design

In each cleared or non-invaded area, we chose five random soil collection points ($n = 30$). In each soil collection point, organic-horizon material was removed and 44 l of soil excavated using a shovel to a depth of 10 cm (modified from Lehnhoff and Menalled, 2013). The soil from each cleared or non-invaded area was thoroughly mixed to obtain a representative soil sample. Half the soil from each cleared or non-invaded area was sterilized to remove soil microbial communities using an autoclave machine at 80 °C in three 60-minute autoclaving periods with 24 hours in-between each period (modified from Meiman et al., 2006), whilst the other half was not (soil microbial communities remained). We chose to use a whole soil treatment rather than inoculating sterile soil with live field soil because we were interested in measuring the total soil-mediated effects of *A. saligna* invasion including both biotic and abiotic components. We acknowledge that the use of soil sterilization through autoclaving and other available methods often presents a challenge because the process is known to alter soil characteristics under some circumstances and this can confound the effect of sterilizing (Troelstra et al., 2001; Perkins et al., 2013). Five soil samples were taken in the autoclaved and non-autoclaved soil from each cleared or non-invaded area, and analysed for % carbon, % nitrogen, nitrate, ammonium and available phosphorus, to determine the effect of autoclaving on soil characteristics that could influence the germination and growth of *P. repens* (Bemlab Pty Ltd, Somerset West).

The soil was taken to a 40 m² greenhouse tunnel with open ends to allow for ventilation, searched for *P. repens* and *E. calycina* seeds, and those that were found were removed. Seedling trays (40 x 27.5 x 11.5 cm) were filled with the soil. Fifty seeds of *P. repens* were planted in each seedling tray. This number was chosen to ensure sufficient germination numbers because germination numbers of *P. repens* are often low (Brown, 1993). The seeds of *P. repens* were pre-treated by soaking in a smoke water solution for 18 hours before planting to improve germination (Brown, 1993). Twenty *E. calycina* seeds per tray were sown into half the number of trays with sterilized or non-sterilized soil from each cleared or non-invaded area, to investigate the competitive or facilitative responses on germination and biomass of *P. repens* when grown together with nitrophilic weedy species. Overall, a fully factorial and completely randomized experimental design was set up in the greenhouse with six replicates per treatment (three study sites x two invasion statuses x two soil microbial community conditions x two nitrophilic weedy species conditions x six replicates = 144 seedling trays).

Each tray was initially watered to field capacity and subsequently given 600 ml of water every two days. The location of trays was rotated in the greenhouse every two weeks. The number of germinating *P. repens* seedlings was recorded every seven days. Ten *E. calycina* seedlings were left in each tray after germination and the rest were weeded out together with non-target species that germinated. *Protea repens* seedlings were harvested five months after planting. The soil from the roots was removed by wet sieving and the roots separated from the shoots. All plant material was oven dried at 60 °C for 48 hours and weighed.

In this study we did not determine how the soil chemistry differed between each cleared area and its reference non-invaded area because results of those differences have been established in a previous study (chapter two). Therefore, we based the legacy of altered soil chemistry in this study on those results.

3.3.4 Statistical analyses

The effects of soil chemical and biotic legacies, and *E. calycina* on *P. repens* root-to-shoot ratio, root and shoot dry mass were analysed as explanatory variables using linear mixed-effects ANOVA models with Satterthwaite's approximation for denominator degrees of freedom using the *lmerTest* package (Kuznetsova et al., 2017) in R version 3.4.3 (R Development Core Team, 2017). Study site was included as a random effect to account for variability between study sites. Residual and Q-Q plots were applied to examine data normality. We first fitted different models with two and three-way interactions of the explanatory variables and included study site as random effects. We compared the different models using the *anova()* function to determine the explanatory variables that best explain variation in *P. repens* root-to-shoot ratio, root and shoot dry mass. We only selected a model if its AICc value was less than the AICc value of all the other models even if it did not include all the explanatory variables. If an additional parameter provides little or no increase in model fit, then the more complex model fits the data equally well or worse as the simpler model. The more complex model with the additional parameter should not be considered for ecological inference, since nothing is explained by the additional complexity (Burnham and Anderson, 2002; Richards, 2008). For each explanatory variable, only one model remained after model selection and we report the results of these best-fitting models. Significant mean differences were separated with Tukey's HSD test using the *lsmeans* package (Lenth, 2016). The effect of soil sterilization on % carbon, % nitrogen, nitrate, ammonium and available phosphorus was also analysed as an explanatory variable using linear mixed-effects ANOVA

models with Satterthwaite's approximation for denominator degrees of freedom using the *lmerTest* package (Kuznetsova et al., 2017). Study site nested with invasion status was included as a random effect to account for variability between the cleared-non-invaded pairs in each study site.

3.4 Results

Germination percentage, root and shoot dry mass of *P. repens* did not significantly differ between altered and native soil chemistry as 'invasion status' was not selected in any best-fitting model for any response variable. Germination percentage of *P. repens* was significantly higher (93%; $p < 0.001$) in the presence of soil microbial communities than in their absence (Figure 3.1). There was a significant interaction between the effects of soil microbial communities and *E. calycina* on root ($p = 0.018$; Figure 3.2) and shoot ($p < 0.001$; Figure 3.3) dry mass of *P. repens*. Root-to-shoot ratio of *P. repens* was significantly higher (29%; $p < 0.001$; Figure 3.5) in the presence of *E. calycina* than in their absence. Soil sterilization led to a significant increase in % nitrogen (38%; $p = 0.017$; Figure 3.4), ammonium (83%; $p < 0.001$; Figure 3.4) and available phosphorus (61%; $p = 0.041$; Figure 3.4).

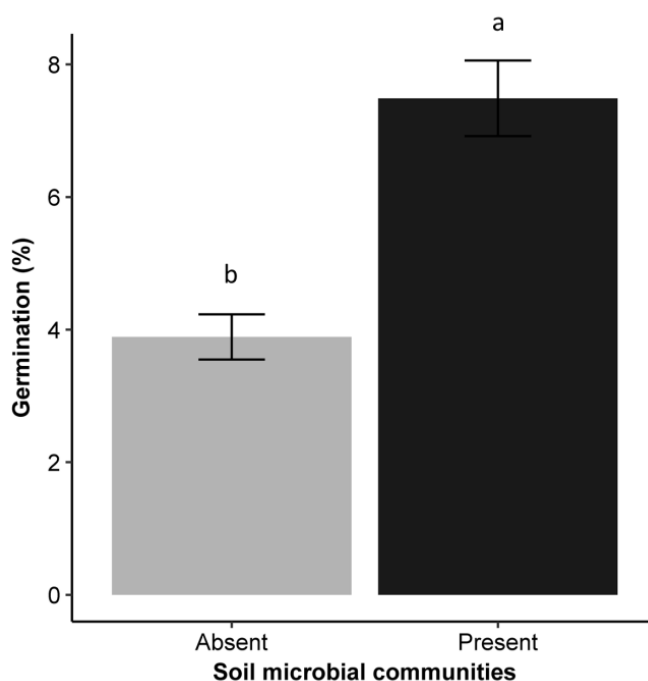


Figure 3.1: The effect of soil microbial communities on the germination percentage of *P. repens*. Mean values with the same letter are not significantly different. Error bars represent \pm SE.

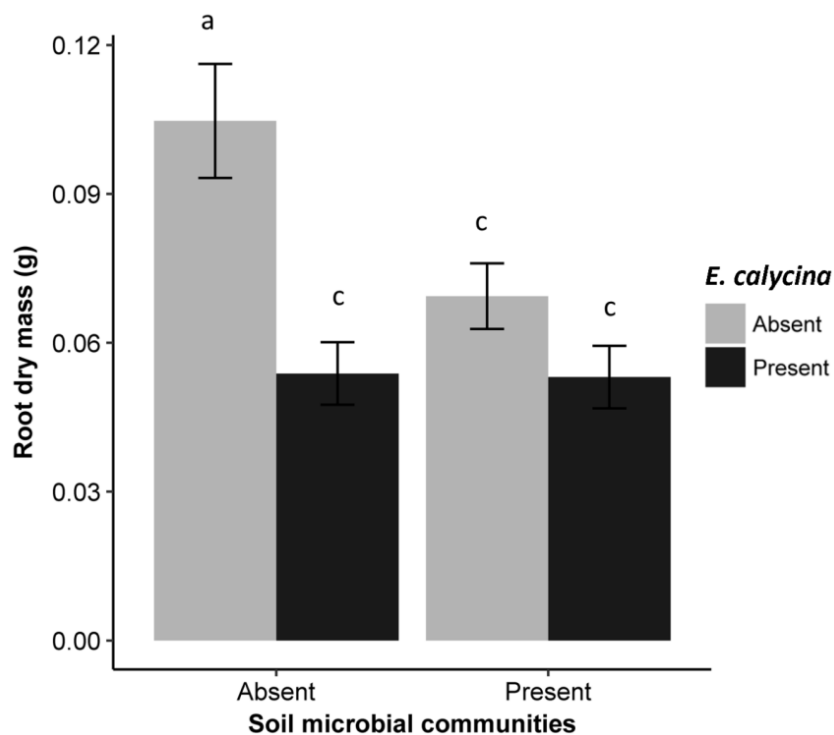


Figure 3.2: The effects of soil microbial communities and *E. calycina* on the root dry mass of *P. repens*. Mean values with the same letter are not significantly different. Error bars represent \pm SE.

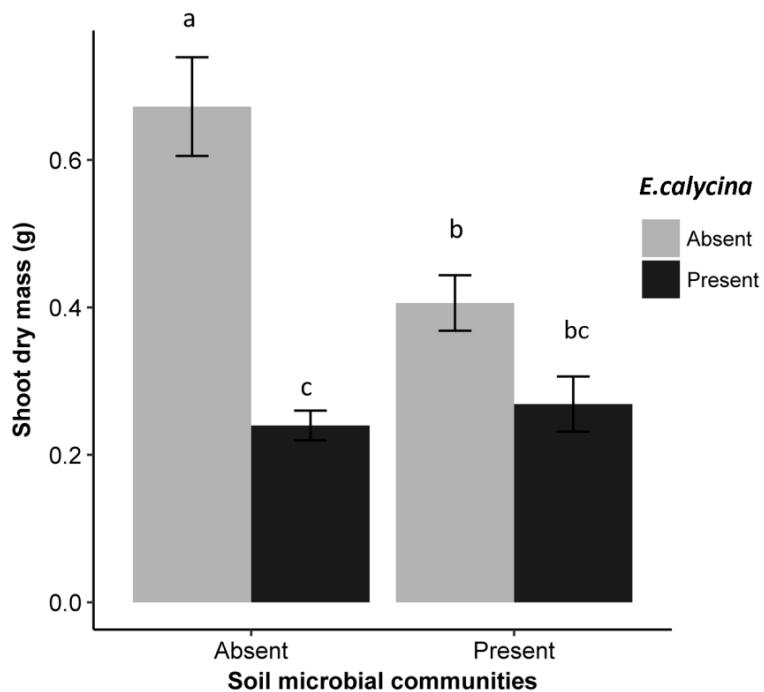


Figure 3.3: The effects of soil microbial communities and *E. calycina* on the shoot dry mass of *P. repens*. Mean values with the same letter are not significantly different. Error bars represent \pm SE.

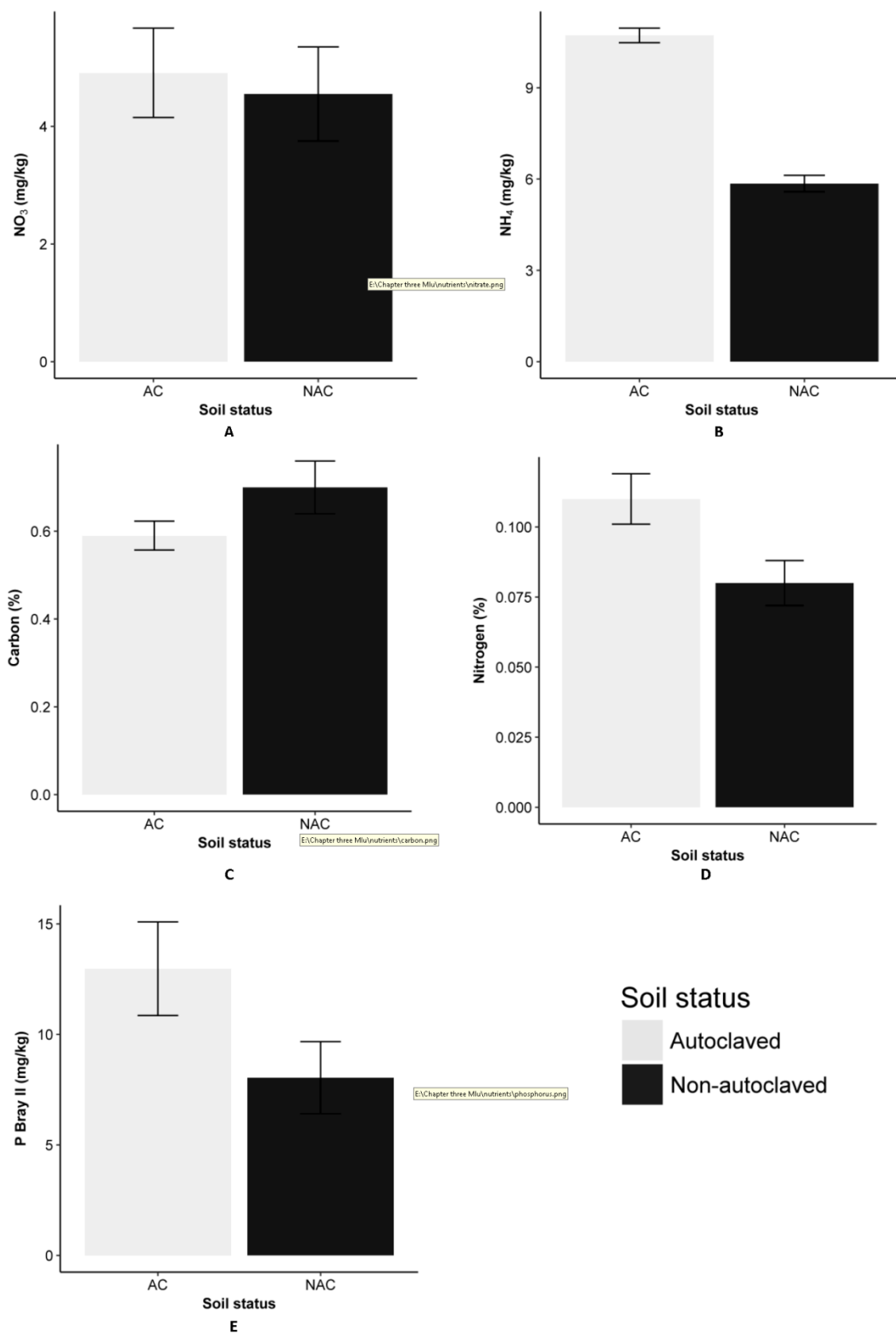


Figure 3.4: Nitrate (A), ammonium (B), % carbon (C), % nitrogen (D) and available phosphorus before and after soil sterilization. Error bars represent \pm SE.

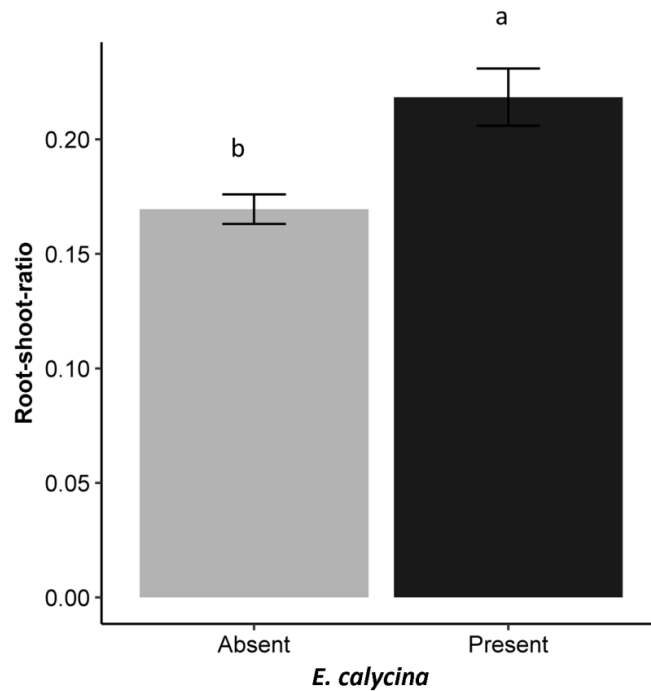


Figure 3.5: The effect of *E. calycina* on the root-to-shoot ratio of *P. repens*. Mean values with the same letter are not significantly different. Error bars represent \pm SE.

3.5 Discussion

Our results suggest that the legacy of altered soil chemistry does not have direct negative consequences on the re-establishment of native proteoid shrubs because the germination and growth of *P. repens* was the same despite differences in soil chemistry between previously invaded and non-invaded areas. Our results support the findings of Musil (1993) and Holmes (2008) which suggested that the growth of *Protea* seedlings is often unaffected by changes in soil chemistry. Furthermore, Holmes (2008) suggested that if total soil available nitrogen is less than double that in native areas, it is unlikely to hinder the establishment of fynbos seedlings (Holmes, 2008). Our findings, put together with those of these other authors, follow the general principle that plants from infertile areas such as South African lowland fynbos, that have relatively low growth and nutrient absorption rates, are often unresponsive to changes in soil characteristics (Grimes and Hunt, 1975; Chapin, 1980). The re-establishment of other typical native fynbos species besides proteoid shrubs, irrespective of whether their seeds were sown or not, was also not negatively affected by the legacy of altered soil chemistry after clearing invasive acacias (Gaertner et al., 2012b; Mostert et al., 2016). The general unresponsiveness of native seedlings to altered soil chemistry has also been observed with other invasive species (e.g. *Psidium cattleianum*, *Macaranga mappia*, *Falcataria*

moluccana, *Melastoma septemnerium* and *Clidemia hirta*; Ostertag et al., 2009; and *Acacia dealbata*; Lorenzo et al., 2017). However, our results conflict with the findings of Witkowski (1991b) which suggested that *P. repens* seedlings displayed an increase in growth in response to increased nutrient availability. We believe that this is a result of differences in the levels of soil nutrients, particularly nitrogen, explored by the two studies. Witkowski (1991b) looked at the effect of growing *P. repens* in soils with nitrogen levels between 50 and 200 g/m², which were much higher than those in this study (chapter two) and in Musil (1993).

Our results suggest that the presence of soil microbial communities can improve *P. repens* germination. Our findings are in line with the results of a study by Lozano et al. (2017) that suggested that soil microbial communities improved the germination of several annual plants in a Mediterranean ecosystem, when compared to soils in which microbial communities were absent. However, our results are contrary to those from several studies where the presence of soil microbial communities either had a negative (Crist and Friese, 1993; O'Hanlen-Manners and Kotanen, 2004) or no effect on native species germination (Rudgers and Orr, 2009). Prior to soil contact, microbial communities can be found on internal seed tissues (Gallery et al., 2007) and the surface (Kremer, 1986). Microbial communities on the seed surface can play a role in preventing pathogen infection when the seeds are in the soil (Kremer, 1987). However, seeds may also become infected by microbes such as ascomycetous fungi after burial in the soil and these may prevent pathogenic attacks on seed contents and/or slow decay of seed enclosing structures (Kluger et al., 2008; Dalling et al., 2011). It is crucial for beneficial microbes to establish first so as to limit subsequent pathogenic infection (Dalling et al., 2011). We suspect that in the presence of soil microbial communities in our study, *P. repens* seeds were infected by beneficial microbes after burial in the soil. These microbes could have played a role in maintaining seed viability, thereby improving germination. However, this requires more testing and direct evidence. We acknowledge that the soil sterilization temperature we used (i.e. 80 °C) could have failed to eliminate all soil microbial communities. Therefore, we urge future studies to use higher temperatures (e.g. 120 °C). The effect of soil microbial communities on germination is largely unexplored and the identity, and function of most seed-associated microbial communities is currently unknown (Lopez-Velasco et al., 2013). More research is required to elucidate the effects of microbial communities on germination rates during restoration projects.

The significant interaction between the effects of soil microbial communities and *E. calycina* on *P. repens* root and shoot dry mass suggests that, in the absence of *E. calycina*, the absence of soil microbial communities leads to significantly higher root and shoot growth than their presence. However, the presence of *E. calycina* overwhelms this beneficial effect and significantly reduces *P. repens* root and shoot growth. We suspect that growth reduction in the presence of soil microbial communities might have been caused by pathogenic microbes that accumulated during invasion and/or disruption of native mutualistic associations, although further research would be required to be able to explain this (Bever et al., 1997; Eppinga et al., 2006; Inderjit and van der Putten, 2010). A study conducted by Mangla et al. (2008) suggested that invasion by *Chromolaena odorata* led to the accumulation of the soil pathogen *Fusarium spp*, which reduced the seedling growth of a native species. However, mycorrhizas are absent from the *Protea* family (Allsopp and Stock, 1993) and proteoid shrubs are considered much less dependent on soil microbial symbionts than most South African fynbos taxa owing to their specialized roots that are efficient in nutrient uptake (Lamont, 1984, Stock et al., 1990). It is also possible that in the absence of soil microbial communities, *P. repens* could have responded to soil nutrient changes imposed by soil sterilization (Fig. 3.5; Perkins et al., 2013). Future studies could use other techniques such as soil inoculation instead of soil sterilization to further elucidate the role of soil microbial communities on *P. repens* germination and growth.

The reduction in *P. repens* root and shoot growth when *E. calycina* was present but soil microbial communities were absent, was most likely a consequence of competition for soil moisture and nutrients (D'Antonio and Vitousek, 1992). Grasses are good competitors against woody species' seedlings because of their rapid uptake of water and nutrients (D'Antonio and Vitousek, 1992) as a result of their dense and shallow root systems (Phillips, 1963). In *Banksia* woodlands in south-west Australia, *E. calycina* invasion is one of the major causes of poor native species establishment because *E. calycina* suppresses native seedling growth by out-competing them for soil moisture (Fisher et al., 2009). Our results suggest that in the presence of soil microbial communities, the presence or absence of *E. calycina* does not make a difference to *P. repens* root and shoot growth. We suspect that pathogenic microbes that were most likely accumulated during invasion, had a significant negative effect on *P. repens* root and shoot growth that was equal to the effect of *E. calycina* presence (Reinhart and Callaway, 2006).

The increase in root-to-shoot ratio (when grown with *E. calycina*) could be a response by *P. repens* seedlings to avoid competition from *E. calycina* for moisture and nutrients, by investing more into root growth, thereby accessing water and nutrients lower in the soil profile (Reynolds and D'Antonio, 1996; Casper and Jackson, 1997). The increase in root-to-shoot ratio is most likely a result of diverting photosynthates into the root system to support lateral root growth towards soil pockets that have more water and nutrients (Drew and Saker, 1975). However, this is not always the case because a shortage of certain minerals such as potassium, magnesium and manganese can lead to reduction in root growth (Ericsson et al., 1996). In a nursery experiment conducted in Denmark, *Quercus robur* seedlings were grown together with grasses, and this led to an increase in their root-to-shoot ratio (Andersen et al., 2000). However, Newton and Cole (1991) suggested that competition was not solely responsible for the alteration of biomass allocation because allocation of photosynthates to roots and shoots can be constant over a wide range of competition intensities.

We acknowledge that growing *P. repens* alone and with *E. calycina* is not an adequate test for competition because an increase in the density of *P. repens* could have given the same outcome (Li and Wilson, 1998). We suggest that future studies should manipulate the densities of both *P. repens* and *E. calycina*. Furthermore, future studies should use a variety of native species (representing the major fynbos guilds) and nitrophilic weedy species (alien and native).

3.6 Conclusions and implications for restoration

Overall, our results suggest that the legacy of altered soil chemistry after clearing invasive *A. saligna* does not necessarily have direct negative consequences on the re-establishment of native proteoid shrubs. While the soil microbial communities after clearing invasive *A. saligna* may have a positive effect on the germination of native proteoid shrubs, the legacy of altered soil microbial communities and presence of nitrophilic weedy species could have negative impacts on their growth. Our results provide some insight on the effect of soil legacies of invasive acacias, and nitrophilic weedy species on native species re-establishment, but given that the results of studies from elsewhere are often inconclusive or contradictory, it is clear that further research is needed.

Since the legacy of altered soil characteristics does not always have a direct negative effect on the re-establishment of native proteoid shrubs, we do not see a need for management interventions to always attempt to restore native soil chemistry. Furthermore, to

our knowledge there is no restoration strategy that can be successfully used to restore overall native soil chemistry, although methods to immobilize elevated nitrogen are available. If elevated nitrogen availability, legacy of altered soil microbial communities and nitrophilic weedy species prove to be barriers to native species re-establishment during restoration; we urge practising restoration ecologists to incorporate management actions such as soil carbon addition, soil microbial treatments and prescribed burning into their restoration programs (chapter one).

3.7 Acknowledgements

Funding for this work was provided by the DST-NRF Centre of Excellence for Invasion Biology and Working for Water Program through their collaborative research project on “Integrated Management of invasive alien species in South Africa”. BWvW thanks the National Research Foundation (grant 109467) for funding. We sincerely thank the City of Cape Town for giving us permission to use their areas for soil collection and Vula Environmental Services for providing us with seeds.

Chapter four: Secondary invasion after clearing invasive *Acacia saligna* in the South African fynbos

4.1 Abstract

It is often assumed that clearing invasive alien species will lead to the dissipation of their negative impacts and recovery of native plant diversity. However, this is often not the case because clearing of primary invasive alien species can lead to secondary invasion by non-target species. We investigated the effects of vegetation type and application of fire during management of biomass after clearing invasive acacias on secondary invasion in the South African fynbos. Furthermore, we determined how these effects change with years after clearing. We sampled vegetation in lowland and mountain fynbos cleared of invasive *Acacia saligna* using the “fell, stack and burn” method. During burning of the stacked slash, the area at the centre of the stack experiences a high severity fire while the area at the edge experiences a low severity fire. After fire, burn scars remain in place of the stacked slash. We sampled in and outside of 80 burn scars over three years after clearing. Overall, we set out to (1) identify species that are secondary invaders; (2) determine whether secondary invader richness and cover differ between where there were high and low severity fires and no fires, and how this changes with years after clearing; and (3) determine whether secondary invader richness and cover differ in and between lowland and mountain fynbos, and how this changes with years after clearing. We identified 32 secondary invader species. Secondary invader richness was lower where there were high severity fires compared to where there were low severity fires (16%) and no fires (15%). Secondary invader cover was lower where there were no fires compared to where there were high severity fires (27%) and low severity fires (30%). Three years after clearing, secondary invader richness and cover remained similar or higher than in the first year, while secondary invader richness was similar between lowland and mountain fynbos. Secondary invader cover was similar between lowland and mountain fynbos up to two years after clearing but was 58% lower in lowland fynbos in the third year. Due to the persistence and abundance of secondary invaders up to three years after clearing at levels similar or higher than in the first year, we conclude that practicing restoration ecologists have to manage these species to ensure successful restoration of native plant diversity.

4.2 Introduction

The human-mediated movement of species from their native ranges to new areas across the world has resulted in most ecosystems being invaded by multiple species (Hobbs et al., 2006). Invasive alien species often have significant negative impacts on recipient native plant diversity, ecosystem function and services, and these impacts differ in direction and magnitude among various levels of ecological complexity (Musil and Midgley, 1990, Musil, 1993; Pejchar and Mooney, 2009; Vilà et al., 2011). Furthermore, the financial cost of invasive species to recipient ecosystems across the globe is staggering (van Wilgen et al., 2001; Pimentel et al., 2005; Vilà et al., 2010). A significant amount of that cost is allocated to clearing alien species from invaded ecosystems (Hulme, 2006).

It is often assumed that manually clearing invasive species will lead to the dissipation of their negative impacts and the recovery of native plant diversity (Wittenberg and Cock, 2005; Grove et al., 2015). This school of thought originates from agriculture where the reduction of a pest often leads to an increase in desirable species (Pearson and Ortega, 2009). However, in complex ecosystems beyond agricultural systems this is often not the case, since invasive species can leave long-lasting legacy effects – i.e. measurable changes to biological, chemical, or physical conditions (Corbin and D’Antonio, 2012). These legacy effects often interact and create barriers that hinder the restoration of desirable native ecosystems in previously invaded areas (chapter one). Furthermore, instead of facilitating the recovery of native plant diversity, clearing of target invasive species (hereafter “primary invaders”) can lead to secondary invasion – i.e. an increase in the abundance of non-target alien species (Pearson et al., 2016). A global meta-analysis of 60 cases from 38 studies conducted by Pearson et al. (2016) found that a decrease in primary invaders due to clearing often leads to secondary invasion, with only a slight recovery in native plant diversity.

Secondary invaders can proliferate in previously invaded areas because clearing primary invaders often (1) creates a “space” for their recruitment from soil seed banks accumulated prior to primary invasion and/or from seeds that migrated from surrounding areas by reducing competition (Grubb, 1977; González-Muñoz et al., 2012; Gioria et al., 2014; Pearson et al., 2016); and/or (2) increases the availability of resources, such as nitrogen from soil legacy effects – i.e. often in the case of nitrogen fixing primary invaders, and light, which facilitates their growth (Loo et al., 2009; chapter two). Furthermore, there is evidence that the method used to clear primary invaders can influence the recovery trajectory and extent of secondary

invasion (Krupek et al., 2016; Pearson et al., 2016). The most selective clearing methods such as classical biological control and hand pulling can lead to more severe cases of secondary invasion than less selective methods (Pearson et al., 2016). Disturbance often influences resource availability by increasing or decreasing it, depending on the disturbance factor and its severity (Davis et al., 2000). For example, eutrophication can lead to elevated nitrogen availability while severe fire can reduce it through volatilization (Davis et al., 2000; Marchante et al., 2009). Secondary invaders are adept at exploiting disturbances, particularly those that lead to increased resource availability (Pearson et al., 2016). Overall, primary invader suppression is often the most important factor that drives secondary invasion (Pearson et al., 2016).

Secondary invaders may also benefit from the post-clearing environment more than native species because of (1) provenance effects – i.e. introduction filters that select for disturbance-adapted traits or release from natural enemies (Buckley and Catford, 2016); (2) the clearing method applied – e.g. use of broadleaf herbicides during clearing can favour the proliferation of secondary invader grasses while suppressing native monocots or dicots (Skurski et al., 2013); and/or (3) anthropogenic activities – e.g. eutrophication can increase nitrogen availability and favour the excessive growth of secondary invaders (Pearson et al., 2016).

Australian acacias have become global invaders since being introduced for a range of ornamental, commercial and subsistence uses (Griffin et al., 2011; Wilson et al., 2011). Approximately 70 Australian *Acacia* species have been introduced to South Africa and at least 14 of these are currently invasive (Richardson et al., 2011). Within South Africa, the fynbos biome is the greatest casualty of Australian *Acacia* invasions (van Wilgen et al., 2011). The “fell, stack and burn” method – i.e. fell primary invaders, stack the slash and allow it to dry before burning it, has been widely applied to reduce the cover of acacias (Holmes et al. 2000; van Wilgen et al., 2012; Ruwanza et al., 2013). During burning of the stacked slash, the area at the centre of the stack experiences a high severity fire while the area at the edge experiences a low severity fire (Ruwanza et al., 2013). After fire, burn scars remain in place of the stacked slash.

There have been significant advances in understanding vegetation dynamics after clearing invasive acacias in the fynbos (Holmes and Cowling, 1997b; Galatowitsch and Richardson, 2005; Reinecke et al., 2008; Blanchard and Holmes, 2008; Gaertner et al.,

2012b). However, given the importance of native plant diversity recovery, research focus has been skewed towards native species dynamics, despite a range of secondary invaders being observed in previously invaded areas (Yelenik et al., 2004; chapter two). Thus, the current knowledge gaps are at least threefold: (1) there has been little work done to identify which species are secondary invaders after clearing invasive acacias in the fynbos. (2) There is little knowledge on the effect of the treatment-related disturbance factor, fire – i.e. no fire, low and high severity, on the extent of secondary invasion after clearing invasive acacias, and whether this changes with years after clearing. (3) There is no knowledge on the effect of fynbos type – i.e. lowland and mountain, on the extent of secondary invasion after clearing invasive acacias, and whether this changes with years after clearing.

We used *Acacia saligna* (Labill.) H.L Wendl. (Fabaceae) invasions in the South African fynbos as case study to address these issues. We addressed the following questions: (1) which species are secondary invaders after clearing invasive *A. saligna*? (2) Does the species richness and cover of secondary invaders after clearing invasive *A. saligna* differ between areas that experienced low and high severity fires and no fires, and does this change with years after clearing? (3) Does the species richness and cover of secondary invaders after clearing invasive *A. saligna* differ in and between lowland and mountain fynbos, and does this change with years after clearing?

4.3 Materials and Methods

4.3.1 Study sites

The study was conducted in two fynbos vegetation types, namely lowland Cape Flats Sand Fynbos (CFSF) and Mountain Sandstone Fynbos (MSF), in the Western Cape Province, South Africa (Rebelo et al., 2006). A Mediterranean-type climate with hot dry summers and cool wet winters is characteristic of both fynbos types (Rebelo et al., 2006). Native vegetation is made up of evergreen shrublands characterised by a mixture of proteoid and ericoid shrubs, and restioid (aphyllous graminoid) growth forms (Rebelo et al., 2006).

We selected Blaauwberg Nature Reserve (33°46'5.16"S; 18°27'10.08"E) to study CFSF, and Glencairn (34°09'24.7"S; 18°24'30.1"E) for MSF. CFSF soil is generally made up of quaternary sand while MSF soil comprises of colluvial sandy loam (Holmes, 2002). Blaauwberg Nature Reserve received a mean annual rainfall of 266 mm during our study period – i.e. 2014 to 2016 (Jacques Kuyler, Blaauwberg Nature Reserve, personal communication), while Glencairn received 775 mm (<http://www.csag.uct.ac.za/current->

seasons-rainfall-in-cape-town/). *Acacia saligna* invasions are fairly common both in the mountains and in the lowlands. Blaauwberg Nature Reserve was invaded by *A. saligna* while most of Glencairn was invaded by *A. saligna* and to a lesser extent by *Acacia cyclops* A.Cunn. ex G.Don (Fabaceae) and *Hakea drupacea* (C.F.Gaertn.) Roem. & Schult. (Proteaceae). Prior to clearing *A. saligna*, both study sites had been invaded for longer than 20 years (75–100% cover). Both study sites were cleared of *A. saligna* and burnt in July 2013, and had been left to recover naturally.

4.3.2 Study design

We selected parts of Blaauwberg Nature Reserve and Glencairn where *A. saligna* was cleared using the “fell, stack and burn” method. In September 2014, we randomly selected 40 burn scars in each study site. Each burn scar was approximately 5×3 m in size. From the centre of each burn scar we ran a transect in a south-east direction to the edge and outside of the burn scar. A permanent plot (1×1 m) was set up perpendicular to the transect at the centre (high severity fire), edge (low severity fire) and outside of the burn scar (no fire; Figure 4.1). Plots at the edge and outside of the burn scars were separated by the same distance as plots at the centre and edge of the burn scar (Figure 4.1). In each plot we recorded all species that were present, classified them according to status (i.e. primary invader, secondary invader or native species; South African National Biodiversity Institute, 2016) and estimated their percentage cover. Furthermore, we estimated the percentage cover of bare ground and litter within the plot. Subsequent samplings were done in September of 2015 and 2016. Plant nomenclature follows South African National Biodiversity Institute (2016).

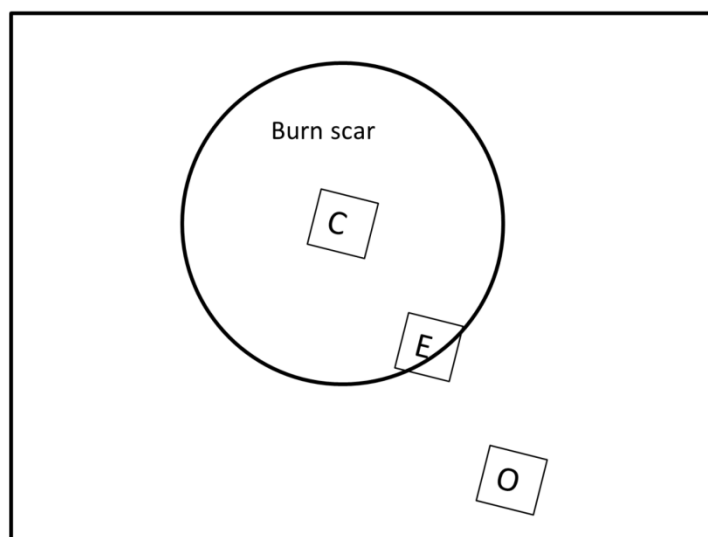


Figure 4.1: Schematic diagram of the sampling protocol, showing the spatial arrangement of plots (1×1 m) used to sample the centre (C; high severity fires), edge (E; low severity fires) and outside of the burn scar (O; no fire). E and O were separated by the same distance as C and E.

4.3.3 Statistical analyses

All statistical analyses were done in R version 3.5.1 (R Development Core Team, 2018). We first converted the percentage cover of secondary invader species to proportions. Residual and Q-Q plots were utilized to examine data normality. We fitted generalized linear mixed models (GLMMs) using *glmmADMB* package (Skaug et al., 2013). We determined the effect of fynbos type (i.e. lowland and mountain), fire (i.e. no fires, low and high severity) and their interactions with years after clearing, on species richness (i.e. Poisson error distribution and log link function) and proportional cover of secondary invaders (i.e. Beta error distribution and logit link function). We included “plot” as a random effect to account for variability between plots. We compared the different models via dredging, an automated procedure implemented by the *MuMIn* package, to identify variables that best explain the variability in species richness and proportion cover of secondary invaders (Barton, 2018). Models were compared using information theoretic (I-T) model procedures based on Akaike's information criterion (AIC; Burnham et al., 2011). The best-fitting model was chosen using the second order AIC value, AICc. The model with the lowest AICc value was chosen as the best-fitting model as it had the smallest information loss, even if it did not include all the explanatory variables and their interactions with years after clearing. For each response variable, only one model remained after model selection. Here we report the results of these

best-fitting models. Significant mean differences were separated with Tukey's HSD test using the *emmeans* package (Lenth, 2018).

4.4 Results

4.4.1 Secondary invader species after clearing invasive *A. saligna* in the fynbos

We documented 32 secondary invader species in areas cleared of invasive *A. saligna* (Table 4.1). These species were grasses (22%), herbs (72%) and shrubs (6%; Table 4.1). We found that 50% of the secondary invader species occurred in both fynbos types while 9% were only found in lowland fynbos and 41% were only found in mountain fynbos (Table 4.1). From the secondary invader species we identified, 91% occurred in areas that experienced high and low severity fires respectively, and 88% were found where there were no fires (Table 4.1). In the first year after clearing, 78% of the secondary invader species were present while 72% occurred in the second year after clearing, and 84% in the third year after clearing (Table 4.1).

Table 4.1: Secondary invaders found where there were high and low severity fires and no fires, up to three years after clearing invasive *A. saligna* in lowland and mountain fynbos. 1 = species present; 0 = species absent.

Species	Growth form	Lowland fynbos	Mountain fynbos	High severity fires	Low severity fires	No fires	1 st Year	2 nd Year	3 rd Year
<i>Avena fatua</i>	Grass	1	1	1	1	1	1	1	1
<i>Briza maxima</i>	Grass	1	1	1	1	1	1	1	1
<i>Bromus diandrus</i>	Grass	1	1	1	1	1	1	1	1
<i>Cirsium vulgare</i>	Herb	0	1	1	1	1	1	1	1
<i>Conyza bonariensis</i>	Herb	0	1	1	1	1	1	1	1
<i>Conyza sumatrensis</i>	Herb	1	1	1	1	1	1	1	1
<i>Echium plantagineum</i>	Herb	1	0	0	1	0	1	0	0
<i>Erodium moschatum</i>	Herb	1	1	1	1	1	1	1	1
<i>Euphorbia helioscopia</i>	Herb	0	1	1	1	1	1	1	1
<i>Fumaria muralis</i>	Herb	1	0	1	1	1	1	1	1
<i>Gamochaeta pensylvanica</i>	Herb	1	0	1	1	1	1	1	1
<i>Geranium purpureum</i>	Herb	1	1	1	1	1	0	0	1
<i>Helminthotheca echioides</i>	Herb	0	1	1	1	1	1	1	1
<i>Hypochaeris radicata</i>	Herb	1	1	1	1	1	1	1	1
<i>Lactuca serriola</i>	Herb	1	1	1	1	1	1	1	1
<i>Lantana camara</i>	Shrub	0	1	0	0	1	1	0	0
<i>Lolium multiflorum</i>	Grass	0	1	1	1	1	1	0	1
<i>Lolium perenne</i>	Grass	0	1	1	1	0	0	0	1

Table 4.1 (continued): Secondary invaders found where there were high and low severity fires and no fires, up to three years after clearing invasive *A. saligna* in lowland and mountain fynbos. 1 = species present; 0 = species absent.

Species	Growth form	Lowland fynbos	Mountain fynbos	High severity fires	Low severity fires	No fires	1 st Year	2 nd Year	3 rd Year
<i>Medicago polymorpha</i>	Herb	0	1	1	1	0	0	1	1
<i>Myoporum tenuifolium</i>	Shrub	0	1	0	0	1	0	0	1
<i>Pennisetum clandestinum</i>	Grass	0	1	1	1	0	0	0	1
<i>Polypogon monspeliensis</i>	Grass	0	1	1	1	1	1	1	0
<i>Raphanus raphanistrum</i>	Herb	1	1	1	1	1	1	1	1
<i>Rapistrum rugosum</i>	Herb	1	1	1	1	1	1	1	1
<i>Silene gallica</i>	Herb	1	1	1	1	1	0	1	0
<i>Sisymbrium orientale</i>	Herb	1	1	1	0	1	1	1	0
<i>Solanum nigrum</i>	Herb	1	1	1	1	1	1	0	1
<i>Sonchus asper</i>	Herb	1	1	1	1	1	1	1	1
<i>Sonchus oleraceus</i>	Herb	0	1	1	1	1	1	1	1
<i>Stellaria media</i>	Herb	1	1	1	1	1	0	1	1
<i>Taraxacum officinale</i>	Herb	1	1	1	1	1	1	1	1
<i>Torilis arvensis</i>	Herb	0	1	1	1	1	1	0	1

4.4.2 *Effect of fire application after clearing invasive *A. saligna* in the fynbos on secondary invader richness and cover, and how it changes with years after clearing*

Secondary invader richness was significantly lower where there were high severity fires compared to where there were low severity fires (16%; $p < 0.001$; 95% confidence interval = 0.08 to 0.30; Figure 4.2) and no fires (15%; $p = 0.001$; 95% confidence interval = 0.07 to 0.29; Figure 4.2). However, secondary invader richness was similar between where there were low severity fires and no fires ($p = 0.85$; 95% confidence interval = -0.12 to 0.09; Figure 4.2). Secondary invader cover was similar between where there were high severity fires and low severity fires ($p = 0.13$; 95% confidence interval = -0.04 to 0.27; Figure 4.2). However, secondary invader cover was significantly lower where there were no fires compared to where there were high severity fires (27%; $p = 0.01$; 95% confidence interval = -0.38 to -0.07; Figure 4.2) and low severity fires (30%; $p < 0.001$; 95% confidence interval = -0.50 to -0.19; Figure 4.2). Secondary invader richness and cover did not change with years after clearing as the “fire severity” and “year” interaction was not selected in any best-fitting model.

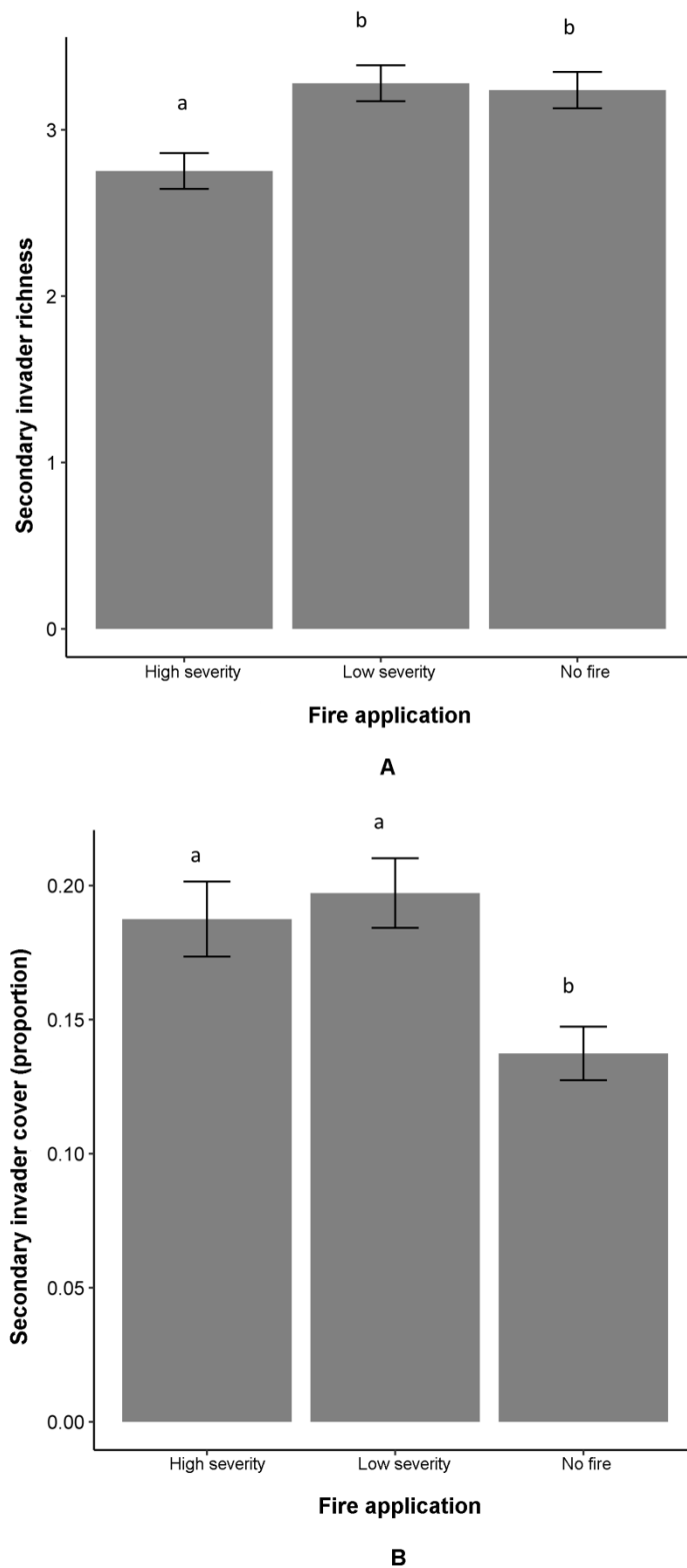


Figure 4.2: Secondary invader richness (A) and cover (B) where there were high and low severity fires and no fires after clearing invasive *A. saligna* in the fynbos. Mean values of secondary invader richness or cover with the same letter are not significantly different. Error bars represent \pm SE.

4.4.3 *Effect of fynbos type on secondary invader richness and cover after clearing invasive A. saligna, and how it changes with years after clearing*

There was a significant interaction between the effects of fynbos type and years after clearing on secondary invader richness (1) in the first and second year after clearing ($p = 0.03$; 95% confidence interval = -0.49 to -0.03) and (2) in the second and third years after clearing ($p = 0.002$; 95% confidence interval = 0.12 to 0.52; Figure 4.3). In lowland fynbos, secondary invader richness significantly increased (64%) in the second year after clearing but did not change significantly thereafter (Figure 4.3). In mountain fynbos, secondary invader richness did not significantly change in all years after clearing (Figure 4.3). Secondary invader richness did not significantly differ between lowland and mountain fynbos in all years after clearing (Figure 4.3).

There was a significant interaction between the effects of fynbos type and years after clearing on secondary invader cover (1) in the first and second year after clearing ($p < 0.001$; 95% confidence interval = -1.16 to -0.51; Figure 4.3); (2) first and third year after clearing ($p < 0.001$; 95% confidence interval = 0.30 to 0.97; Figure 4.3); and (3) second and third year after clearing ($p < 0.001$; 95% confidence interval = 1.17 to 1.76; Figure 4.3). In lowland fynbos, secondary invader cover significantly increased in the second year after clearing (388%; Figure 4.3) but significantly decreased (65%) in the third year to a level similar to that in the first year (Figure 4.3). In mountain fynbos, secondary invader cover significantly increased in the second year after clearing (90%; Figure 4.3) and did not significantly change in third year (Figure 4.3). Furthermore, secondary invader cover did not significantly differ between the lowland and mountain fynbos in the first two years after clearing but was significantly lower (58%; Figure 4.3) in lowland fynbos compared to mountain fynbos in the third year.

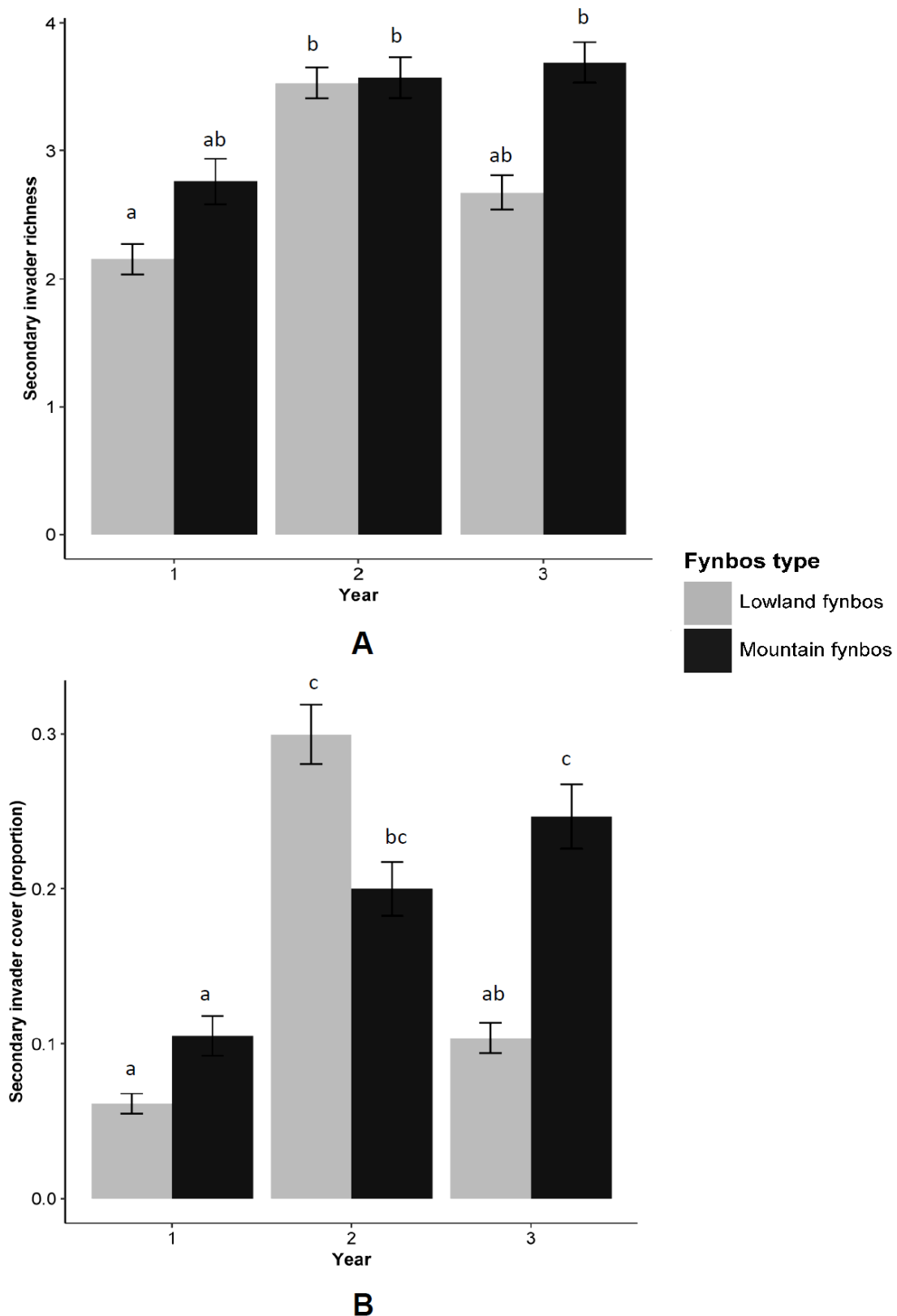


Figure 4.3: Secondary invader richness (A) and cover (B) in lowland and mountain fynbos over three years after clearing invasive *A. saligna*. Mean values of secondary invader richness or cover with the same letter are not significantly different. Error bars represent \pm SE.

4.5 Discussion

4.5.1 *Secondary invader species after clearing invasive A. saligna in the fynbos*

The number of secondary invader species documented in our study is significantly larger than those recorded by previous studies after clearing *A. saligna* invasions (Yelenik et al., 2004) and areas invaded by multiple species in the fynbos (Reinecke et al., 2008; Ruwanza et al., 2013). This is likely because we sampled two types of fynbos over a three year period and therefore were able to capture variation associated with vegetation type and multi-year vegetation monitoring. We do not believe that the size of the area sampled influenced the number of secondary invader species recorded because, compared to our study, Reinecke et al. (2008) sampled a larger area across one habitat type (i.e. only in riparian zones of the fynbos) but recorded fewer species.

4.5.2 *Effect of fire after clearing invasive A. saligna in the fynbos on secondary invader richness and cover, and how it changes with years after clearing*

Secondary invader richness was lower where there were high severity fires compared to where there were low severity fires and no fires, which had similar secondary invader richness. We believe that this could be due to differential recruitment of species from the seed bank (or after dispersal) as a result of the differing fire severities and no fire application. Seed banks of most species are mostly found in the uppermost 3-5 cm (Holmes, 2002; Fourie, 2008) with many small seeds unable to germinate from lower down the soil profile (Bond et al., 1999). During burning of stacks near soil surface temperatures can reach 81 °C in moist soil while in dry soil they can reach 330 °C (Behenna et al., 2008). Therefore, soil seed banks of secondary invaders were likely destroyed by the high severity fires while those that experienced low severity fires were able to survive and recruit. Indeed, germination rates are improved by the lower heat if seeds are fire-cued (Korb et al., 2004; Behenna et al., 2008; Ruwanza et al., 2013; Halpern et al., 2014). Secondary invaders where there were no fires germinate without the inconvenience of having their soil stored seeds destroyed by high severity fires. We suspect that secondary invader richness did not significantly change with years after clearing because a few new species managed to find their way to our study sites while some species were lost each year, possibly as a result of competition (Grubb, 1977).

Severe fires can have significant negative effects on soil structure and microbial communities (Neary et al., 1999; Korb et al., 2004; Cilliers et al., 2005) with negative consequences on the germination of some species (Korb et al., 2004; Ruwanza et al., 2013;

Cilliers et al., 2004). For example, high severity fires can lead to soil water repellency – i.e. reduced water infiltration, which can then reduce seed germination as a result of lowered water availability to the seeds (Scott et al., 1998; Ruwanza et al., 2013).

In contrast to secondary invader richness, secondary invader cover was lower where there were no fires compared to where there were high and low severity fires, which had similar secondary invader cover. This may be a result of elevated nitrogen levels resulting from burning the large amounts of stacked biomass. Dominance of secondary invaders is facilitated by elevated nitrogen availability (Vitousek and Walker, 1989; Le Maitre et al., 2011; chapter two), and burning of stacks can elevate nitrogen availability – i.e. both NH_4^+ and NO_3^- levels (Korb et al., 2004; Fornwalt and Rhoades, 2011). For example, after burning stacks in the Ponderosa Pine in British Columbia, soil nitrate levels were 19 times higher in burnt areas compared to unburned areas (DeSandoli et al., 2016). We accept that some available nitrogen may be lost through volatilization during the burning of stacks (Riggan et al., 1994; Marchante et al., 2009), and with time through leaching, particularly NO_3^- (Dunn et al., 1979). Despite losses in available nitrogen, it is possible that higher than normal levels persist up to three years after clearing (chapter two), thereby leading to a lack of change in secondary invader cover with year after clearing.

4.5.3 Effect of fynbos type on secondary invader richness and cover after clearing invasive A. saligna, and how it changes with years after clearing

In lowland fynbos, secondary invader richness increased in the second year after clearing but did not significantly change afterwards. However, in mountain fynbos, secondary invader richness did not significantly change up to three years after clearing. Furthermore, secondary invader richness was similar between lowland and mountain fynbos up to three years after clearing. We believe that the increase in secondary invader richness came about as a result of new secondary invader species which had not already established in the previously invaded area but existed in surrounding areas, dispersing into the previously invaded area with or without the aid of humans (Pearson et al., 2016). We believe that the lack of significant differences in secondary invader richness between lowland and mountain fynbos shows that both fynbos types are vulnerable to and affected by secondary invasion, with 50% of the documented species occurring at both (Table 4.1).

The increase in secondary invader cover in the second year after clearing in both fynbos types is most likely a result of them growing and increasing their size with time. Our findings

support those by Grove et al. (2015) in areas previously invaded by *Cytisus scoparius* in Washington, U.S.A, where secondary invader cover increased with time up to 22 months after clearing.

We suspect that the decrease in secondary invader cover observed in lowland fynbos in the third year after clearing was caused by an increase in native plant diversity recovery. Native species cover in our lowland fynbos study site increased by 81% in the third year after clearing, while the mountain fynbos study site only experienced a 28% increase (M.M. Nsikani, unpublished data). Several studies have suggested that the recovery of native plant diversity can suppress secondary invader dominance through competitive interactions (Reinecke et al., 2008; Sher et al., 2010; Gaertner et al., 2012b; Pearson et al., 2016; González et al., 2017). It is worth noting that the establishment of one native species may not be enough to suppress secondary invader dominance but an assortment of native species may be required. For example, during active restoration of a site invaded by *Pennisetum clandestinum*, one of the secondary invaders documented in our study, sowing native *Leucadendron coniferum* did not suppress its dominance (Gaertner et al., 2012b).

4.6 Conclusions and implications for restoration

Restoration ecologists need to be aware that a range of secondary invaders may be dominant and persistent after clearing invasive acacias. Furthermore, application of fire after clearing invasive acacias appears to favour the dominance of secondary invaders. Secondary invasion appears not be habitat specific – i.e. occurred in both lowland and mountain fynbos, and can persist up to three years after clearing at levels similar to or higher than the first year. Management of secondary invaders might be site or vegetation type specific. Therefore, depending on practical considerations, we urge practicing restoration ecologists to manage secondary invasion through actions such as herbicide or graminicide application, grazing, manual weeding, mowing, prescribed burning, soil nitrogen management, soil solarisation and weed mats (chapter one).

4.7 Acknowledgements

Funding for this work was provided by the DST-NRF Centre of Excellence for Invasion Biology and Working for Water Program through their collaborative research project on “Integrated Management of invasive alien species in South Africa”. We are sincerely grateful to Dale Slabbert, Hannah Vogt, Luke Potgieter, Mashudu Mashau, Nombuso Ngubane,

Phumudzo Ramabulana, Suzaan Kritzinger-Klopper, Ulrike Irlich and the City of Cape Town Green Jobs Unit for assisting with vegetation sampling. We would like to thank the City of Cape Town for giving us permission to use their areas for vegetation sampling.

Chapter five: Soil nitrogen availability and competitive interactions shape secondary invasion and weedy native species dominance after clearing invasive *Acacia saligna*

5.1 Abstract

Invasive Australian acacias often alter the soil chemistry of ecosystems they invade. Clearing invasive acacias often fails to restore native soil chemistry but instead leaves a legacy of elevated soil nitrogen availability. These legacy effects can facilitate the dominance of secondary invaders and weedy native species that are adapted to high-nitrogen conditions. Given that multiple secondary invader species often dominate areas previously invaded by acacias, negative species interactions can be common as a result of competition between the species. We conducted plant growth chamber and greenhouse experiments to investigate the extent to which a range of soil nitrate levels, representing those associated with non-invaded areas and after clearing invasive acacias, influence the germination and growth of five secondary invaders and weedy native species. Furthermore, we determined whether two secondary invaders, *Avena fatua* and *Briza maxima*, germinate and grow better in monocultures or mixtures. Growth of secondary invaders and weedy native species decreased with a decline in soil nitrate levels. *Avena fatua* root and shoot dry mass did not significantly differ between monocultures and mixtures. However, *B. maxima* root and shoot dry mass was lower (50% and 60% respectively) in mixtures than in monocultures. We conclude that elevated soil nitrate levels after clearing invasive *A. saligna* promote the growth of secondary invaders and weedy native species. Therefore, if these elevated levels are managed, the impacts of secondary invaders and weedy native species can be avoided.

5.2 Introduction

The extensive movement of species beyond their natural distribution ranges has resulted in a significant number of them becoming invasive (Ewel et al., 1999). Invasive Australian acacias form a notable proportion of global plant invasions (Richardson and Rejmánek, 2011) and are found in various habitats such as Mediterranean ecosystems and forests (Le Maitre et al., 2011; González-Muñoz et al., 2012), and across geographical regions such as Africa and North America (Richardson et al., 2011).

Invasive acacias often exert a range of negative ecological impacts and transform ecosystems they invade (Richardson et al., 2000; Richardson and van Wilgen, 2004). They can change the soil chemistry (Musil, 1993; Marchante et al., 2008; Lazzaro et al., 2014),

alter soil microbial community composition and function (Marchante et al., 2008; Boudiaf et al., 2013; Crisóstomo et al., 2013; Lorenzo et al., 2013), exclude native species through competition while forming dense woodlands (Le Maitre et al., 2011), and create feedbacks that favour their dominance (Gaertner et al., 2014).

Management actions such as clearing the invasive acacias, removing their litter, reducing their seed banks, removing *Acacia* seedlings that germinate after clearing the adult plants, re-introduction of native species through seed and/or vegetative propagules, are often applied to reduce their negative impacts and to restore viable native plant communities in previously invaded areas (Richardson and Kluge, 2008; Le Maitre et al., 2011; van Wilgen et al., 2011; Gaertner et al., 2012a). It is common to assume that the negative impacts of invasive species will decrease after such management interventions (Wittenberg and Cock, 2005). However, negative impacts of invasive acacias can remain as legacy effects that persist for long periods after clearing the invasive species (Marchante et al., 2009; Rodríguez-Echeverría et al., 2013; Maoela et al., 2016; chapter two). Legacy effects include quantifiable differences in the biological, soil physical or chemical characteristics that remain after clearing the invasive species (*sensu* Corbin and D'Antonio, 2004; Corbin and D'Antonio, 2012).

The legacy of altered soil chemistry, particularly elevated nitrogen availability, has been shown to persist up to ten years after clearing invasive acacias (Marchante et al., 2009; chapter two) and facilitate secondary invasion and weedy native species dominance (Yelenik et al., 2004; Marchante et al., 2009; Le Maitre et al., 2011; Gaertner et al., 2012b; González-Muñoz et al., 2012; Fill et al., 2018). Secondary invasion is an increase in the quantity of non-target alien plant species after clearing the target invasive species (Pearson et al., 2016) while weedy native species dominance is an increase in the quantity of native species that are not typically found and wanted in the target area, and have detectable impacts (Pyšek et al., 2004). Seeds of secondary invaders and weedy native species are often present in the soil seed bank of the cleared areas or disperse to such sites from surrounding areas (Pearson et al., 2016; chapter three). Secondary invasion and weedy native species dominance often hinder the restoration of viable native plant communities (chapter one).

When discussing the role of soil nitrogen availability after clearing invasive acacias in facilitating secondary invasion and weedy native species dominance, studies often focus on secondary invaders and weedy native species independently (e.g. Yelenik et al., 2004; Pearson et al., 2016; chapter three) rather than jointly (but see chapter two), despite that they

often occur on the same sites (e.g. Fill et al., 2018). There is value in investigating the factors that promote secondary invasion and weedy native species dominance, particularly on the same sites, because this gives insights into the requirements for their management and furthers the understanding of vegetation dynamics after clearing invasive acacias.

Secondary invaders and weedy native species can establish feedbacks that maintain elevated soil nitrogen availability through the production of rapidly decomposing litter characterised by a low carbon to nitrogen ratio and increased nitrogen mineralization rates (Ehrenfeld, 2003; Perry et al., 2010). However, plant species often differ in their ability to assimilate available soil nitrogen (de Graaf et al., 1998). Therefore, the extent to which elevated soil nitrogen availability is reflected in the growth and dominance of different species often varies (Yelenik et al., 2017).

In ecosystems with multiple alien species, negative interactions can be common, thereby reflecting the important role played by competition (Kuebbing and Nuñez, 2015). Negative interactions show that some alien species may be inhibiting the spread of other alien species in ecosystems and they could be an unexplored form of biotic resistance (Zenni and Nuñez, 2013). Despite the probability of co-existence among secondary invaders being high, their negative interactions are relatively understudied (Pearson et al., 2016). Investigating negative interactions among secondary invaders is crucial for understanding their distribution and abundance (Kuebbing and Nuñez, 2015). Knowledge of secondary invader distribution and abundance can inform management requirements after clearing invasive acacias.

Here, we explore the extent to which soil nitrogen availability after clearing invasive acacias, particularly nitrate levels, influences the germination and growth of five secondary invaders and weedy native species, and how this differs between species. Also, we investigate negative interactions between two common secondary invaders, particularly whether they germinate and grow better in monocultures or mixtures. We explore these issues using *Acacia saligna* (Labill.) H. L. Wendl. (Fabaceae) invasions in the South African lowland fynbos as case study. The germination phase was included in this study because studies from elsewhere have reported that seed germination, particularly in weeds, can be influenced by soil nitrate levels (Roberts and Smith, 1977; Goudey et al., 1988; Bell et al., 1999).

5.3 Materials and Methods

5.3.1 Study area

In this study, soil nitrate levels were determined from empirical measurements in non-invaded areas and those previously invaded by *A. saligna* in the Cape Flats Sand Fynbos (CFSF) of the Western Cape Province in South Africa (chapter two). The soils of the CFSF are broadly classified as well-drained aeolian acidic sands and their natural chemistry is characterised by low soil nitrogen levels (Musil, 1993).

5.3.2 Study species

A wide range of secondary invaders and weedy native species often dominate areas previously invaded by *A. saligna* in the fynbos (Yelenik et al., 2004; Gaertner et al., 2012b; chapter two; chapter three; chapter four). On the basis of seed availability and ease of collection, we selected five of the 32 secondary invaders that are often found after clearing invasive *A. saligna* to be study species (chapter four): (i) *Avena fatua* (L.) (Poaceae), (ii) *Briza maxima* (L.) (Poaceae), (iii) *Bromus diandrus* (Roth.) (Poaceae), (iv) *Hypochaeris radicata* (L.) (Asteraceae), and (v) *Raphanus raphanistrum* (L.) (Brassicaceae). We also included *Ehrharta calycina* (Sm.) (Poaceae) as a study species because it is one of the most common weedy native species found after clearing invasive *A. saligna* (Yelenik et al., 2004). We selected more secondary invaders than weedy native species because the species richness and percentage cover of secondary invaders is often significantly greater than weedy native species dominance in previously invaded CFSF areas (M.M. Nsikani, unpublished data). We specifically chose to investigate negative interactions between *A. fatua* and *B. maxima* because they are the most common secondary invaders after clearing invasive *A. saligna* on our study species list (chapter four).

5.3.3 Study design

5.3.3.1 Seed collection and pre-germination treatment

Seeds of each study species were collected from six populations of wild plants in late 2016 and early 2017 while they were producing seed. Seeds were then stored in brown paper bags under dry conditions at room temperature until experiments were conducted in July 2017. Seeds of all study species, except *R. raphanistrum*, did not require pre-germination treatment to break seed dormancy. Before the start of the experiments, *R. raphanistrum* seeds

were soaked in hot water, i.e. 50 °C, for 15 minutes to break seed dormancy (L.C Campbell, University of Sydney, personal communication).

5.3.3.2 Soil nitrate levels

We chose soil nitrate levels following measurements in chapter two, taken in the CFSF, viz. 1, 3, 7.5 and 12 mg/kg, to investigate the effect of soil nitrate level on study species performance – i.e. germination (number and percentage) and growth (root and shoot dry mass). The 1 mg/kg soil nitrate level represented that found in non-invaded areas (chapter two). The 3, 7.5 and 12 mg/kg soil nitrate levels represent the lowest, median and highest levels often found in areas previously invaded by *A. saligna* up to ten years after clearing (chapter two). Clearing invasive *A. saligna* elevates soil nitrate levels but these levels can decrease with years after clearing due to factors such as leaching (chapter two).

“Haifa Cal” ($\text{Ca}(\text{NO}_3)_2$), a fully water soluble fertilizer (Haifa Chemicals Ltd, Haifa, Israel), was used as the source of nitrate. Soil nitrate levels were established through proportional fertigation (Sne, 2006) using nutrient solutions by adding 0.13, 0.4, 0.96 and 1.53 g Haifa Cal/20 l of water respectively (Table 5.1). The phosphorus level in this study followed the measurements from chapter two taken in non-invaded CFSF areas. “Haifa MKP” (KH_2PO_4), a fully water soluble fertilizer (Haifa Chemicals Ltd, Haifa, Israel), was used as a source of phosphorus and potassium and 0.25 g was uniformly added to each soil nitrate level nutrient solution (Table 5.1). The magnesium level in this study followed the measurements by Musil (1993) in non-invaded CFSF areas. “Haifa BitterMag” (MgSO_4), a fully water soluble fertilizer (Haifa Chemicals Ltd, Haifa, Israel) was used a source of magnesium and sulfur and 82 g was uniformly added to each soil nitrate level nutrient solution (Table 5.1). Overall each soil nitrate level nutrient solution contained the six essential macronutrients required for plant growth (Barker and Pilbeam, 2015).

Table 5.1: Nutrient composition of each soil nitrate level nutrient solution.

Nutrient	Concentration (milligrams)			
Nitrates	1	3	7.5	12
Calcium	1.3	4	8.9	14.6
Phosphorus	3	3	3	3
Pottasium	3.5	3.5	3.5	3.5
Magnesium	41	41	41	41
Sulfur	46	46	46	46

5.3.3.3 Germination experiment

We used 100 × 15 mm polystyrene Kord-Valmark™ petri dishes lined with two 90 mm Munktell™ filter paper disks that were initially soaked in 1.5 ml 0.1% Benomyl® fungicide to reduce fungal growth. For the first part of the experiment – i.e. without interspecific competition, for each soil nitrate level, five seeds per species were placed in each petri dish as far apart as possible to reduce the chances of the seeds touching each other (six species × four soil nitrate levels × five replicates = 120 petri dishes). For the second part of the experiment – i.e. performance of *A. fatua* and *B. maxima* in monocultures and mixtures, for each nitrate level, varying amounts of *A. fatua* and *B. maxima* were placed in each petri dish following a replacement design (Table 5.2) as far apart as possible, to reduce the chances of the seeds touching each other (11 seed densities × four soil nitrate levels × five replicates = 220 petri dishes). A replacement design for two species consists of a pure stand of each species and a range of mixtures in which species are sown at proportions P and $1-P$ of their pure stand densities respectively. Overall plant density remains constant (Connolly, 1988).

Petri dishes were transferred to a Sanyo™ incubator set to alternating temperatures of 21° C for 10 hours of light and 10° C for 14 hours of darkness in random stacks of five petri dishes. These conditions reflect winter conditions – the main field germination season in the fynbos (Yelenik et al., 2004). The seeds in each petri dish were watered with 3 ml of the respective soil nitrate level nutrient solution every three days, the petri dish stacks were

randomly reorganised and the number of seeds that germinated was counted. In this study, germination is defined as the clear and unobstructed emergence of the radicle, or seedling root, from the seed coat (Bicksler, 2011). The germination experiment was discontinued after 12 days when there were no signs of any new seeds germinating.

5.3.3.4 Growth experiment

Four litre, 20 cm-diameter polystyrene pots were filled with sterile sand (Cape Silica Suppliers, Cape Town, South Africa) and taken to a greenhouse tunnel (Forestry Department, Stellenbosch University). For the first part of the experiment – i.e. without interspecific competition, for each soil nitrate level, five seeds per species were sown at a depth of 2cm in each pot as far apart as possible to reduce the chances of the seeds touching each other (six species \times four soil nitrate levels \times five replicates = 120 pots). One plant per pot was left after germination while the rest were weeded out. For the second part of the experiment – i.e. performance of *A. fatua* and *B. maxima* in monocultures and mixtures, twice the required amounts of *A. fatua* and *B. maxima* seeds were evenly sown (to reduce clumping of plants) at a depth of 2 cm in each pot following a replacement design (Table 5.2) as far apart as possible (11 seed densities \times four soil nitrate levels \times five replicates = 220 pots). The required numbers of plants were left after germination while the rest were weeded out (Table 5.2).

Each pot was initially watered to field capacity with the respective nutrient solution and subsequently given 600ml of the nutrient solution every two days. Due to the relatively high level of drainage in each pot as a result of the numerous drainage holes, we assumed that no nutrient solution was carried over to the next watering period. The location of each pot was randomly rotated in the greenhouse every two weeks. The plants were harvested five months after planting. The soil from the roots was removed by wet sieving, and the roots separated from the shoots. All plant material was oven dried at 60 °C for 48 h and weighed.

Table 5.2: Seed and plant densities of *A. fatua* and *B. maxima* (X) used for germination and growth experiments following a replacement design to determine their performance in monocultures and mixtures in the selected soil nitrate levels.

<i>A. fatua</i>	<i>B. maxima</i>				
	0	5	10	15	20
0		X	X	X	X
5	X			X	
10	X		X		
15	X	X			
20	X				

5.3.4 Statistical analyses

Statistical analyses were carried out in R version 3.4.3 (R Development Core Team, 2017). Residual and Q-Q plots were applied to examine data normality. The effect of soil nitrate level on the germination proportion of all species was analysed as an explanatory variable using generalized linear mixed models and “species” was included as a random effect to account for variability among the study species (function = *glmer*; family = binomial). The interaction between the effects of soil nitrate level and study species on the germination proportion of all species was analysed as an explanatory variable using generalized linear models (function = *glm*; family = binomial). The effect of soil nitrate level on root and shoot dry mass of all species was analysed as an explanatory variable using linear mixed-effects models and “species” was included as a random effect to account for variability among the study species (function = *lmer*). In the event of significant differences, to determine the differences in root and shoot dry mass of all species between soil nitrate levels, we re-cast “soil nitrate level” with a different reference level each time until all the levels had been included. The interaction between the effects of soil nitrate level and study species on root and shoot dry mass of all species was analysed as an explanatory variable using generalized linear models (function = *glm*). The effects of growth status – i.e. monocultures or mixtures, number of plants and their interaction, on their germination

percentage, root and shoot dry mass of *A. fatua* or *B. maxima* were analysed as explanatory variables using linear mixed-effects models (function = *lmer*). “Soil nitrate level” was included as a random effect to account for variability among the soil nitrate levels.

5.4 Results

5.4.1 Effect of soil nitrate levels on the performance of all species

Germination proportion did not significantly differ between soil nitrate levels and all species responded similarly to soil nitrate levels (Table 5.3). Root dry mass at 1 mg/kg was lower than at 3 (55%), 7.5 (75%) and 12 (72%) mg/kg (Table 5.3; Figure 5.1). Root dry mass at 3 mg/kg was significantly lower than at 7.5 (46%) and 12 (38%) mg/kg (Table 5.3; Figure 5.1). Root dry mass did not significantly differ between 7.5 and 12 mg/kg (Figure 5.1). There was a negative significant interaction between the effects of species and soil nitrate level on the root dry mass of *A. fatua* and *E. calycina*, and *A. fatua* and *H. radicata* (Table 5.3). *Avena fatua* root dry mass was higher than that of *E. calycina* and *H. radicata* at 1 (50% and 3200% respectively), 3 (89% and 1430% respectively), 7.5 (161% and 1141% respectively) and 12 (197% and 352% respectively) mg/kg.

Shoot dry mass at 1 mg/kg was significantly lower than at 3 (63%), 7.5 (77%) and 12 (82%) mg/kg (Table 5.3; Figure 5.1). Shoot dry mass at 3 mg/kg was significantly lower than at 7.5 (37%) and 12 (51%) mg/kg (Table 5.3; Figure 5.1). Shoot dry mass at 7.5 mg/kg was significantly lower (23%) than at 12 mg/kg (Table 5.3; Figure 5.1). There was a negative significant interaction between the effects of species and soil nitrate level on the shoot dry mass of *A. fatua* and *E. calycina*, and *B. diandrus* and *E. calycina* (Table 5.3). *Avena fatua* shoot dry mass was higher than that of *E. calycina* at 1 (75%), 3 (102%), 7.5 (81%) and 12 (72%) mg/kg. *Bromus diandrus* shoot dry mass was higher than that of *E. calycina* at 1 (98%), 3 (40%) and 12 (104%) mg/kg. However, *E. calycina* shoot dry mass 21% higher than that of *Bromus diandrus* at 7.5 mg/kg.

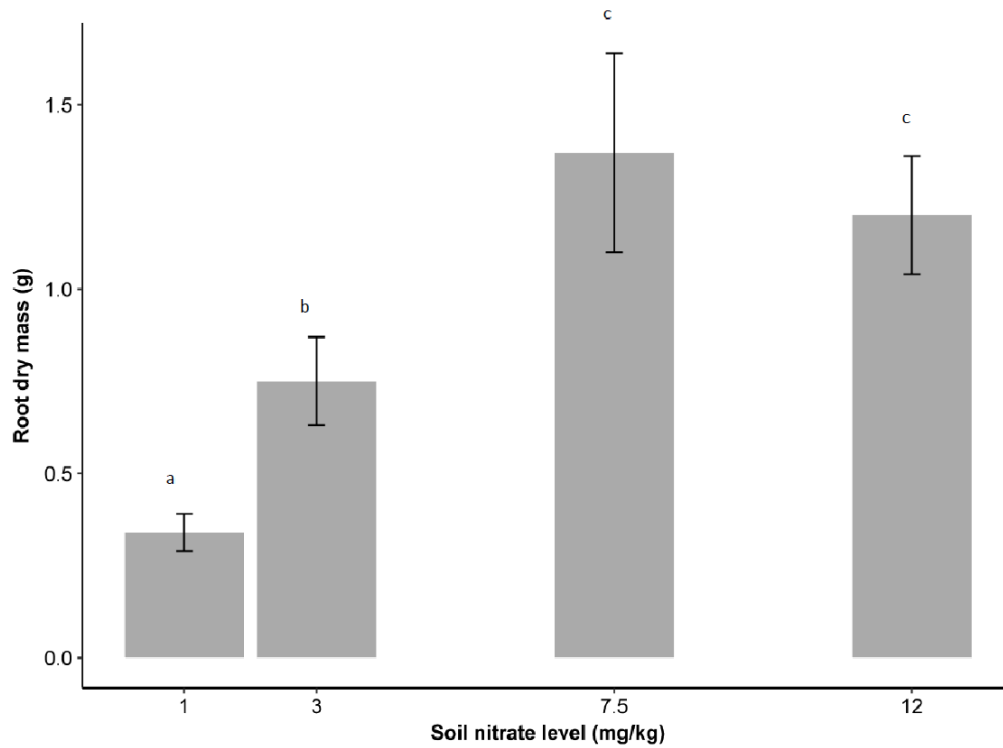
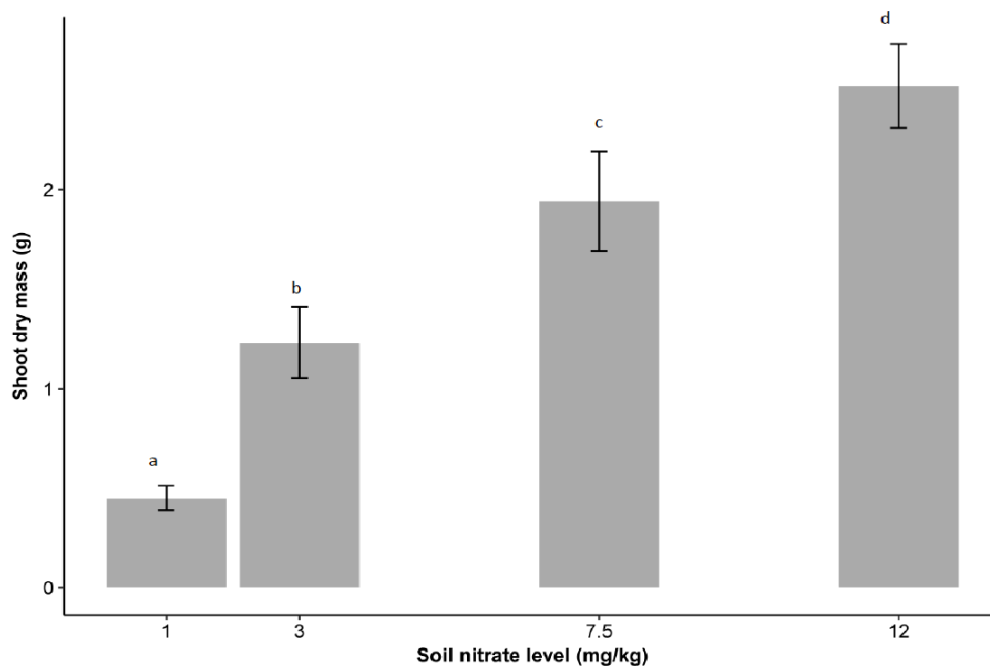
**A****B**

Figure 5.1: Root (A) and shoot (B) dry mass of all study species in the selected soil nutrient levels. Mean values of root or shoot dry mass with the same letter are not significantly different. Error bars represent \pm SE.

Table 5.3: Significant results of generalized linear mixed models, generalized linear models and linear mixed-effects models of the effects of soil nitrate level, species and their interaction on germination proportion, root and shoot dry mass of all study species. Interaction between variables indicated as X. Non-significant results are in appendix A.

Explanatory variable	Response variable	Reference species/conditions	Response species/conditions	Estimate	SE	p-value	t-value	95% confidence interval
Soil nitrate level	Root dry mass	-	-	0.08	0.01	-	5.33	(0.05 to 0.11)
		1 mg/kg	3 mg/kg	0.41	0.15	-	2.72	(0.12 to 0.70)
		1 mg/kg	7.5 mg/kg	0.98	0.15	-	6.44	(0.69 to 1.28)
		1 mg/kg	12 mg/kg	0.79	0.16	-	4.95	(0.48 to 1.10)
		3 mg/kg	7.5 mg/kg	0.57	0.15	-	3.71	(0.27 to 0.87)
		3 mg/kg	12 mg/kg	0.38	0.16	-	2.37	(0.07 to 0.70)
Study species X Soil nitrate level		<i>A. fatua</i> : Soil nitrate level	<i>E. calycina</i> : Soil nitrate level	-0.12	0.04	<0.01	-2.73	(-0.20 to -0.03)
		<i>A. fatua</i> : Soil nitrate level	<i>H. radicata</i> : Soil nitrate level	-0.11	0.05	0.04	-2.11	(-0.21 to -0.01)
Soil nitrate level	Shoot dry mass	-	-	0.17	0.01	-	12.49	(0.15 to 0.20)
		1 mg/kg	3 mg/kg	0.79	0.15	-	5.33	(0.50 to 1.08)
		1 mg/kg	7.5 mg/kg	1.41	0.15	-	9.40	(1.12 to 1.71)
		1 mg/kg	12 mg/kg	2.01	0.16	-	12.77	(1.71 to 2.32)
		3 mg/kg	7.5 mg/kg	0.62	0.15	-	4.09	(0.33 to 0.92)
		3 mg/kg	12 mg/kg	1.22	0.16	-	7.68	(0.91 to 1.53)
		7.5 mg/kg	12 mg/kg	0.60	0.16	-	3.72	(0.29 to 0.91)
Study species : Soil nitrate level		<i>A. fatua</i> : Soil nitrate level	<i>E. calycina</i> : Soil nitrate level	-0.09	0.04	0.04	-2.08	(-0.17 to -0.01)
		<i>B. diandrus</i> : Soil nitrate level	<i>E. calycina</i> : Soil nitrate level	-0.10	0.05	0.04	-2.13	(-0.19 to -0.01)

5.4.2 Performance of *A. fatua* and *B. maxima* in monocultures and mixtures

Germination percentage of *A. fatua* and *B. maxima* did not significantly differ between monocultures and mixtures (Table 5.4). *Avena fatua* root and shoot dry mass did not significantly differ between monocultures and mixtures (Table 5.4). *Briza maxima* root growth was significantly lower (50%) in mixtures than in monocultures (Table 5.4; Figure 5.2). *Briza maxima* shoot growth was significantly lower (60%) in mixtures than in monocultures (Table 5.4; Figure 5.2). There was no significant interaction between the effects of growth status and number of plants, on root and shoot dry mass, and germination of *A. fatua* and *B. maxima* (Table 5.4).

Table 5.4: Significant results of linear mixed-effects models of the effects of growth status, number of plants and their interaction on germination percentage and root and shoot dry mass of *A. fatua* and *B. maxima*. Non-significant results are in appendix B.

Explanatory variable	Response variable	Reference species/conditions	95% confidence interval
<i>B. maxima</i> growth status (i.e. monocultures vs. mixtures)	Root dry mass	Growth status	(-0.03 to -0.01)
<i>A. fatua</i> growth status (i.e. monocultures vs. mixtures)	Shoot dry mass	Number of plants	(-0.02 to -0.01)
<i>B. maxima</i> growth status (i.e. monocultures vs. mixtures)		Growth status	(-0.09 to -0.02)

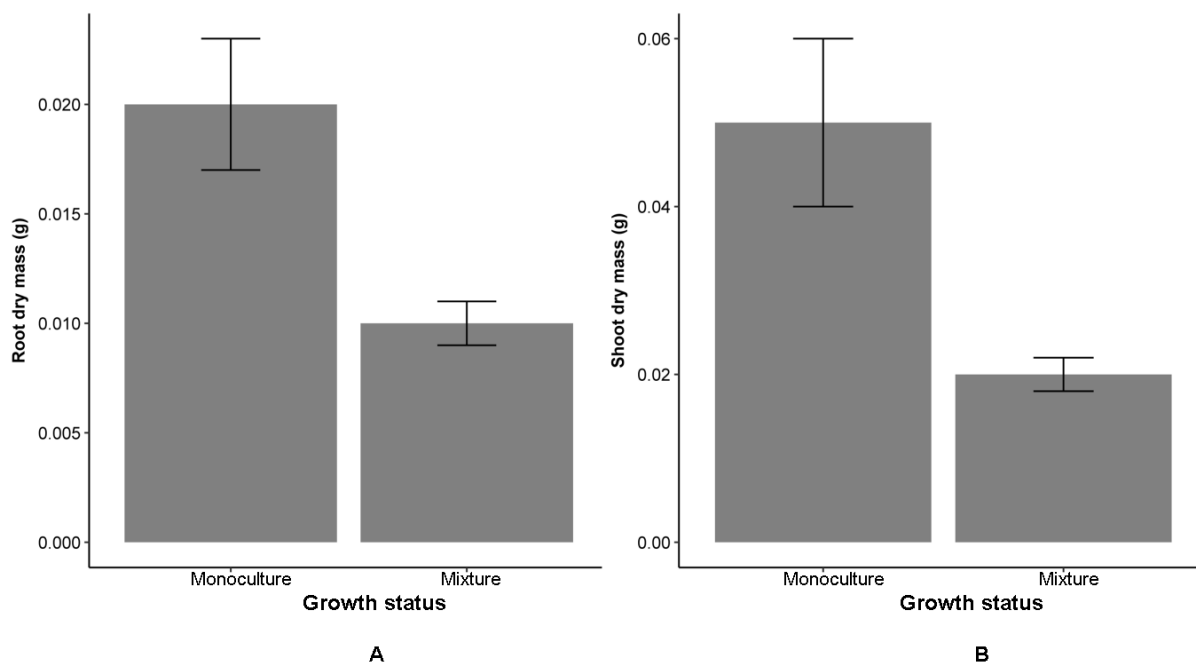


Figure 5.2: *Briza maxima* root (A) and shoot (B) dry mass in monocultures and mixtures. Error bars represent \pm SE.

5.5 Discussion

5.5.1 Effect of soil nitrate levels on species performance

Our findings are in line with the results of a study by Alpert and Maron (2000) in a Mediterranean ecosystem, where reduction in nitrogen availability led to a decrease in secondary invader biomass, with grasses being the most affected. Our results also support the findings by Yelenik et al. (2004) in the South African fynbos, where weedy native species biomass was lower in soil with low compared to high nitrogen availability.

Our results, put together with those of these other authors, follow the general principle that secondary invasion and weedy native species dominance are facilitated by elevated soil nitrogen availability because the species are often nitrophilic by nature (Maron and Jefferies, 1999; Marchante et al., 2008, 2009; Le Maitre et al., 2011; Pearson et al., 2016). Furthermore, our results and those of these other authors suggest that elevated soil nitrogen availability facilitates secondary invasion and weedy native species regardless of whether secondary invaders and weedy native species occur together or alone. The growth of secondary invaders and weedy native species most likely benefits from increases in soil nitrogen availability because it leads to higher foliar nutrient concentrations that elevate photosynthetic capacity, which in turn prompts a rise in their growth (Vitousek and Sanford

Jr, 1986). The higher growth for some species compared to others at the same soil nitrate level is probably caused by several interacting species-specific factors such as higher nutrient acquisition and photosynthesis rates (Clarkson, 1985; Tilman, 1986).

Root growth potentially remains unchanged despite a decline in soil nitrate levels from 12 to 7.5 mg/kg as a result of secondary invaders and weedy native species prioritizing the allocation of photosynthates to root growth over shoot growth to capture more soil nitrates (Ericsson, 1995; Poorter and Nagel, 2000). However, this pattern is most likely not maintained when soil nitrate levels are at 7.5 mg/kg or lower because their decline severely limits the supply of soil nitrates to produce photosynthates such that both root and shoot growth are affected (Ericsson, 1995; Poorter and Nagel, 2000).

5.5.2 Performance of *A. fatua* and *B. maxima* in monocultures and mixtures

Our results suggest that some secondary invaders grow better in monocultures than mixtures, while others have no preference. Therefore, some secondary invaders can exclude others in mixtures through competition for limiting resources such as available nitrogen. It is also possible that *A. fatua* reduced the growth of *B. maxima* in mixtures through the influence of allelochemicals (Tinnin and Muller, 1972). *Avena fatua* produces at least five phytotoxic phenolic acids and these have been shown to inhibit the growth of other species, particularly annuals (Tinnin and Muller, 1972; Liu et al., 2016). It is also possible that *A. fatua* was able to succeed in mixtures at the expense of *B. maxima* through size asymmetries created by initial conditions, differences in maximum nutrient uptake rates (on a per gram root basis) or faster growth rates (Wedin and Tilman, 1993).

We do not think that *A. fatua* could have limited *B. maxima* growth through the resource reduction mechanism of competition (R^* model; Tilman, 1982) because our study was short-term and we maintained soil nitrate levels using nutrient solution additions (Wedin and Tilman, 1993). In short-term competition studies plants cannot maintain limiting nutrients at critically low levels because extensive root systems have not had adequate time to develop (Wedin and Tilman, 1993). Furthermore, we believe that *A. fatua* could not have limited *B. maxima* growth in our study through higher nitrogen use efficiency because they are both C_3 grasses (Milton, 2004), and are therefore characterised by similar nitrogen-use efficiency (Niu et al., 2008).

5.6 Conclusions

We conclude that growth of secondary invaders and weedy native species increases with an increase in soil nitrate levels. However, some secondary invaders and weedy native species grow better than others at the same soil nitrate level. Despite similarity in growth form, some secondary invaders are less competitive than others, and therefore grow better in monocultures than in mixtures. Therefore, some secondary invaders can exclude others in mixtures through competition for limiting resources such as available nitrogen. Restoration ecologists need to be aware that dominance of secondary invaders and weedy native species can be reduced by managing soil nitrate levels. Depending on the scale and context, practicing restoration ecologists elsewhere have incorporated soil carbon addition, litter removal, prescribed burning and establishing species adapted to low N availability, into their restoration programs to manage soil nitrate levels (chapter one).

5.7 Acknowledgements

Funding for this work was provided by the DST-NRF Centre of Excellence for Invasion Biology and Working for Water Program through their collaborative research project on “Integrated Management of invasive alien species in South Africa”. We are sincerely grateful to Suzaan Kritzinger-Klopper and Mashudu Mashau for assisting with seed collection.

Chapter six: Conclusion and implications for restoration

This study had several aims (Fig. I), with the end goal of providing management recommendations to improve restoration outcomes, particularly in relation to secondary invaders and weedy native species, since these species present significant barriers to restoration. Literature review, soil sample analyses, growth chamber and greenhouse experiments were utilised to achieve these aims. This chapter summarises the major findings of this study and presents management actions to improve restoration outcomes after clearing invasive *A. saligna*.

6.1 Conclusion

Altered soil microbial communities, depleted native soil seed banks, elevated N status, secondary invasion and weedy native species dominance, and reinvasion are potential barriers to restoration presented by soil legacy effects of invasive alien N₂-fixing woody species (chapter one). Management actions that can potentially be used to address these barriers to restoration include carbon addition, litter removal, soil microbial treatments, establishing species adapted to low N levels, prescribed burning, classical biological control, grazing, mowing, herbicide or graminicide application, manual weeding, soil N management, soil solarisation, weed mats, native species reintroduction, and the use of nurse plants (chapter one). Despite addressing distinct barriers to restoration, these management actions are rarely applied in combination (chapter one).

Clearing invasive *A. saligna* does not restore overall native soil chemistry and the legacy of altered overall soil chemistry may persist up to ten years after clearing (chapter two). Furthermore, clearing invasive *A. saligna* elevates soil nitrate levels and these can remain higher than in invaded and non-invaded areas up to ten years after clearing (chapter two). *Acacia saligna*'s soil chemical legacies identified in chapter two do not necessarily have direct negative consequences on the re-establishment of native proteoid shrubs (chapter three). However, they can facilitate weedy native species dominance (chapter five). *Acacia saligna*'s biotic legacies and weedy native species can, however, have negative consequences on the growth of native proteoid shrubs (chapter three). The soil chemical legacies of *A. saligna* identified in chapter two and application of fire after clearing can facilitate secondary invasion (chapter four and five).

A range of species, i.e. up to 32, can be secondary invaders after clearing invasive *A. saligna* (chapter four). Secondary invasion after clearing invasive *A. saligna* appears not to be habitat specific, and can persist up to three years after clearing at levels similar to or higher than the first year (chapter four). However, some secondary invaders and weedy native species identified in chapter three and four grow better than others at the same soil nitrate levels (chapter five). Despite similarity in growth form, some secondary invaders identified in chapter four are less competitive than others, such that they grow better in monocultures than mixtures (chapter five). Therefore, some secondary invaders identified in chapter four can exclude others in mixtures through competition for limiting resources such as soil nitrates (chapter five). A decrease in soil nitrate levels and lack of fire after clearing invasive *A. saligna* could decrease the dominance of secondary invaders and weedy native species (chapter five).

6.2 Implications for restoration

Practicing restoration ecologists are encouraged to combine management actions into an integrated management effort to improve restoration outcomes (chapter one). Active restoration by planting native species could, over time, return soils to previous conditions after clearing invasive *A. saligna* (chapter two). Many species adapted to low soil nitrogen availability (e.g. typical native fynbos species) can (i) immobilize nitrogen and slow its cycling using various mechanisms, such as producing relatively recalcitrant litter with a high carbon to nitrogen ratio; and (ii) restore ecosystem function, when they successfully re-establish (Perry et al., 2010). Strategies to reduce soil nitrogen availability such as biomass removal, carbon addition, establishing species adapted to low N availability, grazing, prescribed burning and topsoil removal and their efficacy have been reviewed by Perry et al. (2010). Therefore, practicing restoration ecologists are encouraged to consider these strategies when managing areas previously invaded by *A. saligna* if elevated soil nitrogen availability proves to be a barrier to restoration (chapter two).

Acacia saligna's soil chemical legacies do not always have a direct negative effect on the re-establishment of native proteoid shrubs (chapter three). Therefore, there is no need for management interventions to always attempt to restore native soil chemistry when *A. saligna*'s soil chemical legacies prove not to be a barrier to restoration (chapter three). However, soil biotic legacies, secondary invasion and weedy native species dominance need to be managed if they prove to be barriers to restoration (chapter three, four and five).

Depending on practical considerations, *Acacia saligna*'s soil biotic legacies can be managed through the application of soil microbial treatments and removal of *Acacia* litter (chapter one and three). Practicing restoration ecologists can also incorporate herbicide or graminicide application, grazing, manual weeding, mowing, prescribed burning, soil nitrogen management, soil solarisation and weed mats into their restoration programs (chapter one, two, three, four and five).

6.3 Future research

Overall, this study furthers the knowledge on barriers to restoration after clearing invasive alien N₂-fixing woody species, particularly *A. saligna*. Such knowledge is invaluable given the global demand for the restoration of viable native plant communities in previously invaded areas. This study identified that restoration outcomes can be improved by combining management actions into an integrated management effort. However, there is need for further research on the efficacy of different integrated combinations of management approaches to address barriers to restoration presented by soil legacy effects invasive alien N₂-fixing woody species, particularly *A. saligna*.

This study monitored *A. saligna*'s soil legacy effects up to ten years after clearing and secondary invasion up to three years after clearing. However, future research should go beyond these timelines to capture long-term trends of soil legacy effects and secondary invasion after clearing invasive *A. saligna*. Furthermore, this study investigated interactions between two secondary invaders but future research should investigate more species to expand the knowledge on secondary invader dynamics after clearing invasive *A. saligna*. To test the effect of weedy native species on proteoid shrubs, this study grew *P. repens* alone and with *E. calycina*. However, this is not an adequate test for competition because an increase in the density of *P. repens* could have given the same outcome. Therefore, future research should manipulate the densities of both *P. repens* and *E. calycina*. Furthermore, future studies should use a variety of native species (representing the major fynbos guilds), secondary invaders and weedy native species.

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Appendix A: Non-significant results of generalized linear mixed models, generalized linear models and linear mixed-effects models of the effects of soil nitrate level, species and their interaction on germination proportion, root and shoot dry mass of all study species. Interaction between variables indicated as X.

Explanatory variable	Response variable	Reference species/conditions	Response species/conditions	Estimate	SE	p-value	t-value	95% confidence interval
Soil nitrate level	Germination proportion	-	-	0.06	0.10	0.52	-	(-0.13 to 0.27)
Study species X Soil nitrate level		<i>A. fatua</i> X Soil nitrate level	<i>B. maxima</i> X Soil nitrate level	0.02	0.35	0.96	-	(-0.77 to 0.78)
		<i>A. fatua</i> X Soil nitrate level	<i>B. diandrus</i> X Soil nitrate level	0.07	0.47	0.89	-	(-1.00 to 1.54)
		<i>A. fatua</i> X Soil nitrate level	<i>E. calycina</i> X Soil nitrate level	0.04	0.29	0.90	-	(-0.68 to 0.71)
		<i>A. fatua</i> X Soil nitrate level	<i>H. radicata</i> X Soil nitrate level	0.07	0.47	0.89	-	(-1.00 to 1.54)
		<i>A. fatua</i> X Soil nitrate level	<i>R. raphanistrum</i> X Soil nitrate level	0.10	0.30	0.73	-	(-0.62 to 0.79)
		<i>B. maxima</i> X Soil nitrate level	<i>B. diandrus</i> X Soil nitrate level	0.05	0.45	0.91	-	(-0.98 to 1.50)
		<i>B. maxima</i> X Soil nitrate level	<i>E. calycina</i> X Soil nitrate level	0.02	0.25	0.94	-	(-0.56 to 0.55)
		<i>B. maxima</i> X Soil nitrate level	<i>H. radicata</i> X Soil nitrate level	0.05	0.45	0.91	-	(-0.98 to 1.50)
		<i>B. maxima</i> X Soil nitrate level	<i>R. raphanistrum</i> X Soil nitrate level	0.09	0.26	0.73	-	(-0.50 to 0.63)
		<i>B. diandrus</i> X Soil nitrate level	<i>E. calycina</i> X Soil nitrate level	-2.829e-02	4.055e-01	0.94	-	(-1.45 to 0.94)

Appendix A (continued): Non-significant results of generalized linear mixed models, generalized linear models and linear mixed-effects models of the effects of soil nitrate level, species and their interaction on germination proportion, root and shoot dry mass of all study species. Interaction between variables indicated as X.

Explanatory variable	Response variable	Reference species/conditions	Response species/conditions	Estimate	SE	p-value	t-value	95% confidence interval
Study species X Soil nitrate level	Germination proportion	<i>B. diandrus</i> X Soil nitrate level	<i>H. radicata</i> X Soil nitrate level	-5.560e-16	5.486e-01	1.00	-	(-1.53 to 1.53)
		<i>B. diandrus</i> X Soil nitrate level	<i>R. raphanistrum</i> X Soil nitrate level	3.895e-02	4.095e-01	0.92	-	(-1.39 to 1.02)
		<i>E. calycina</i> X Soil nitrate level	<i>H. radicata</i> X Soil nitrate level	0.03	0.41	0.94	-	(-0.94 to 1.45)
		<i>E. calycina</i> X Soil nitrate level	<i>R. raphanistrum</i> X Soil nitrate level	0.07	0.18	0.70	-	(-0.28 to 0.43)
		<i>H. radicata</i> X Soil nitrate level	<i>R. raphanistrum</i> X Soil nitrate level	3.895e-02	4.095e-01	0.92	-	(-1.39 to 1.02)
Soil nitrate level	Root mass dry	7.5 mg/kg	12 mg/kg	-0.19	0.16	-	-1.17	(-0.51 to 0.13)
Study species X Soil nitrate level		<i>A. fatua</i> X Soil nitrate level	<i>B. maxima</i> X Soil nitrate level	-0.08	0.05	0.11	-1.62	(-0.18 to 0.02)
		<i>A. fatua</i> X Soil nitrate level	<i>B. diandrus</i> X Soil nitrate level	-0.05	0.05	0.30	-1.05	(-0.14 to 0.04)
		<i>H. radicata</i> X Soil nitrate level	<i>R. raphanistrum</i> X Soil nitrate level	0.04	0.05	0.45	0.75	(-0.06 to 0.14)
	Shoot mass dry	<i>A. fatua</i> X Soil nitrate level	<i>B. maxima</i> X Soil nitrate level	-0.06	0.05	0.21	-1.28	(-0.16 to 0.03)

Appendix A (continued): Non-significant results of generalized linear mixed models, generalized linear models and linear mixed-effects models of the effects of soil nitrate level, species and their interaction on germination proportion, root and shoot dry mass of all study species. Interaction between variables indicated as X.

Explanatory variable	Response variable	Reference species/conditions	Response species/conditions	Estimate	SE	p-value	t-value	95% CI
Study species X Soil nitrate level	Shoot dry mass	<i>A. fatua</i> X Soil nitrate level	<i>B. diandrus</i> X Soil nitrate level	0.01	0.05	0.80	0.25	(-0.08 to 0.10)
		<i>A. fatua</i> X Soil nitrate level	<i>H. radicata</i> X Soil nitrate level	-0.09	0.05	0.06	-1.90	(-0.19 to 0.00)
		<i>A. fatua</i> X Soil nitrate level	<i>R. raphanistrum</i> X Soil nitrate level	-0.07	0.04	0.10	-1.67	(-0.16 to 0.01)
		<i>B. maxima</i> X Soil nitrate level	<i>B. diandrus</i> X Soil nitrate level	0.07	0.05	0.16	1.41	(-0.03 to 0.18)
		<i>B. maxima</i> X Soil nitrate level	<i>E. calycina</i> X Soil nitrate level	-0.03	0.05	0.61	-0.51	(-0.12 to 0.07)
		<i>B. maxima</i> X Soil nitrate level	<i>H. radicata</i> X Soil nitrate level	-0.03	0.05	0.58	-0.55	(-0.14 to 0.08)
		<i>B. maxima</i> X Soil nitrate level	<i>R. raphanistrum</i> X Soil nitrate level	-0.01	0.05	0.84	-0.21	(-0.11 to 0.09)
		<i>B. diandrus</i> X Soil nitrate level	<i>H. radicata</i> X Soil nitrate level	-0.10	0.05	0.05	-1.98	(-0.21 to 0.00)
		<i>B. diandrus</i> X Soil nitrate level	<i>R. raphanistrum</i> X Soil nitrate level	-0.08	0.05	0.08	-1.76	(-0.18 to 0.01)

Appendix A (continued): Non-significant results of generalized linear mixed models, generalized linear models and linear mixed-effects models of the effects of soil nitrate level, species and their interaction on germination proportion, root and shoot dry mass of all study species. Interaction between variables indicated as X.

Explanatory variable	Response variable	Reference species/conditions	Response species/conditions	Estimate	SE	p-value	t-value	95% CI
Study species X Soil nitrate level	Shoot dry mass	<i>E. calycina</i> X Soil nitrate level	<i>H. radicata</i> X Soil nitrate level	-0.01	0.05	0.91	-0.11	(-0.10 to 0.09)
		<i>E. calycina</i> X Soil nitrate level	<i>R. raphanistrum</i> X Soil nitrate level	0.01	0.04	0.74	0.33	(-0.07 to 0.10)
		<i>H. radicata</i> X Soil nitrate level	<i>R. raphanistrum</i> X Soil nitrate level	0.02	0.05	0.69	0.40	(-0.08 to 0.12)

Appendix B: Non-significant results of linear mixed-effects models of the effects of growth status, number of plants and their interaction on germination percentage and root and shoot dry mass of *A. fatua* and *B. maxima*. Interaction between variables indicated as X.

Explanatory variable	Response variable	Reference species/conditions	95% confidence interval
<i>A. fatua</i> growth status (i.e. monocultures vs. mixtures)	Germination percentage	Number of plants	(-0.01 to 0.00)
		Growth status	(-0.05 to 0.05)
		Number of plants X Growth status	(0.00 to 0.00)
<i>B. maxima</i> growth status (i.e. monocultures vs. mixtures)		Number of plants	(0.00 to 0.00)
		Growth status	(-0.10 to 0.03)
		Number of plants X Growth status	(0.00 to 0.01)
<i>A. fatua</i> growth status (i.e. monocultures vs. mixtures)	Root dry mass	Number of plants	(-0.01 to 0.00)
		Growth status	(-0.09 to 0.04)
		Number of plants X Growth status	(0.00 to 0.01)
<i>B. maxima</i> growth status (i.e. monocultures vs. mixtures)	Root dry mass	Number of plants	(0.00 to 0.00)
		Number of plants X Growth status	(0.00 to 0.00)
<i>A. fatua</i> growth status (i.e. monocultures vs. mixtures)	Shoot dry mass	Growth status	(-0.20 to 0.07)
		Number of plants X Growth status	(-0.01 to 0.01)
<i>B. maxima</i> growth status (i.e. monocultures vs. mixtures)		Number of plants	(0.00 to 0.00)
		Number of plants X Growth status	(0.00 to 0.01)