Biochemical properties of soil in forest restoration: a case study of native vegetation within *E. globulus* plantations in the south west of Western Australia.

Katarzyna Marta Safianowicz

Submitted in total fulfilment of the requirements of the degree of Doctor of Philosophy

December 2013

School of Biological Sciences and Biotechnology
Murdoch University
Declaration

This is to certify that:

1. The thesis comprises only my original work towards the PhD,
2. Due acknowledgement has been made in the text to all other material used,
3. The thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Katarzyna Safianowicz

December 2013
Acknowledgements

This work was funded by the Murdoch University Research Scholarship with top-up from the CRC for Forestry.

I gratefully acknowledge the following people:

My supervisors: Dr Robert Archibald, for his infinite patience, kindness and support with fieldwork, statistics and writing; Professor Giles Hardy and Associate Professor Treena Burgess.

Plantation companies involved in the CRC for Forestry in 2008-2012: Timbercorp, Great Southern, APFL, and their staff, especially Bob and Justine Edwards, Geoff Rolland, Tom Rouse and Peter English, for access to the field sites and assistance with fieldwork. The Department of Agriculture and Food (DAFWA) and Discovery Inn in Albany for facilitation of fieldwork.

Dr Mamoru Matsuki for classical music, statistics and food.

Anne Griffiths for the most important conversations.

Staff and students at Murdoch University, for sharing laboratory spaces and reagents, access to equipment and for stimulating discussions, especially Dr Katinka Ruthrof, Paco Tovar, Dr Brad Evans, Dr Trudy Paap, Dr Bill Dunstan, Monique Sakalidis, Peter Scott, David Berryman, Dr Joe Fontaine, Dr Nari Williams, Harry Eslick, Maria Waters, Jose and Tim.

Jarek Bialkowski, Terri Jones and Sonia Aghighi for fieldwork assistance.

Staff and students of the ERIE group at University of Western Australia, especially Prof. Richard Hobb, Dr Mike Perring, Dr Kris Hulvey, Rebecca Parsons, Tim Morald and Martha Orozco-Aceves.

Staff, members and students of the CRC for Forestry, especially Dr Neil Davidson, Dr Peter Kanowski, Prof. Brad Potts, Dr Peter Grimbacher, Dr Jie-Lian Beh and Dr Sandra Hawthorne.

My family and friends for continuous support, especially Karolina Albrychiewicz, Jarek Bialkowski, Sarah Ong and Mirja Guldner.

Belen Silva, Leanne McRay and Antonio Cerrone for tango.

Staff and students at the University of Sydney, especially Dr Tina Bell, A/Prof. Michael Kertesz, Carolina, Jessica, Iona, Lori, Michael, Neil, Fei and Nanako.

Dr Matthew Hall for friendly advice, support and chats over coffee in difficult times during writing.

Dr Merlin Thomas and Dr Diane Webster for understanding and encouragement.
Finally, the developers of the R project, especially packages “vegan” and “FactoMineR”, for assistance with data analysis through voluntary contributions to open-source software. Without them most of this work would not have been possible.
Abstract

Soil microbial communities play an essential role in nutrient cycling and influence functioning of the ecosystems though taking part in feedback mechanisms that shape plant communities. Anthropogenic influences on vegetation can result in changes in the activity of soil microbes, which is reflected in soil chemical and biochemical properties, for example nutrient content, enzymatic activity or respiration. Such information about microbial function in soils can enhance research focusing on land use change, degradation and restoration.

Large-scale deforestation of land for agricultural use in the south west of Western Australia (SWWA) and subsequent artificial afforestation of resulting pastureland with Eucalyptus globulus plantations created a mosaic of native vegetation remnants, exotic forest and pastures. Remnants of native woodland in such a fragmented landscape are prone to decline, often as a result of the legacy of pastoral uses of the neighbouring land, such as grazing by livestock. Degradation of the remnants undermines the efforts to preserve native biodiversity of the region. Restoration undertaken to improve their condition is usually by herbicide application and planting of native understorey species. To date, little research is available to guide these efforts towards efficient and successful use of scarce resources allocated to rehabilitation in land management projects. The main objective of this study was to determine the usefulness of soil biochemical properties in assessing land degradation and restoration treatments in native vegetation remnants enclosed in E. globulus plantations in SWWA. It was hypothesised that differences in soil biochemical properties detected using microplate assays would allow assessment and monitoring of soil following land use change and potential of restoration treatments. A second objective was to determine which out of the restoration or degradation treatments had the most influence on soil chemical properties as well as on weed cover, native vegetation growth and regeneration in remnant vegetation. The
hypotheses tested were that: a) soil nutrient enrichment would be a more influential factor contributing to degradation than clearing and incursion of exotic ryegrass in the remnants with native understorey and b) herbicide application and mulching with plantation harvest residue would improve survival and growth of seedlings planted for revegetation in remnants with exotic understorey. This information is important for informing the plantation companies responsible for managing remnant woodland in their estates, particularly those with forest product certification. A third objective was to find chemical and biochemical properties of soil that were both descriptors and predictors of processes involved in disturbance and restoration to investigate the possibility of developing an inexpensive tool for monitoring the condition of remnant vegetation and assessing the suitability of putative restoration treatments. It was hypothesised that soil functional diversity based on soil chemical and biochemical properties could be used as an indicator of land condition.

The first study revealed that while soils from five land conditions common in the landscapes of SWWA differed in their chemical and biochemical properties, soil physical properties were more influential than soil chemical and biochemical properties. The second study showed that addition of fertilizer, but not clearing of native vegetation or incursion of exotic ryegrass, altered the soil biochemistry in remnant vegetation with native understorey. It also revealed that soil alkaline phosphomonoesterase and $\beta$-glucosidase activity and soil respiration in response to succinic, cinnamic and $\alpha$-ketoglutaric acids were related to the outcomes of disturbance of both native and exotic vegetation. The results of subsequent studies showed, contrary to the expectations, that mulching with plantation harvest residue increased soil nutrient levels, especially potassium and nitrate concentration. Herbicide application and mulching improved the growth and survival of native seedlings planted for revegetation; however, there were species-specific responses to each treatment. Analysis of the relationships among soil chemical and biochemical properties revealed that soil nitrogen pools and pH were most
influential on soil biochemistry. Functional diversity varied among soils from different land condition, but not among the experimental treatments applied. Soil nitrate, ammonium, potassium and sulphur, alkaline phosphomonoesterase activity and N-mineralization potential together with respiration in response to imidazole, thiamine and the organic acids: succinic, cinnamic and α-ketoglutaric, were useful in discerning land conditions and experimental treatments.

Microplate assays for soil biochemical properties provided information useful in discriminating among land conditions; however, they were not sensitive enough to clearly detect the effects of all experimental interventions. The usefulness of a soil functional diversity approach as an index for disturbance was tentatively confirmed; however, more work is required to assure its’ robustness and wider applicability.

The importance of soil nitrogen and pH in altering soil biochemical properties found in this study is consistent with previous research. Soil nitrogen was more important than soil phosphorus in maintaining exotic plant incursion in remnant vegetation with native understorey, suggesting that managing nitrogen pools by avoiding excess fertilizer application and encouraging denitrification could be a useful strategy to minimise the weed load in remnant vegetation, one of the biggest impediments to restoration. Mulching with plantation harvest residue with occasional application of glyphosate controlled exotic weeds as effectively as the herbicide-only treatment, while benefitting the survival and growth of the native plant seedlings more, probably through increasing moisture retention and providing nutrients. The choice of restoration treatments have to be considered in conjunction with the choice of plant species used for revegetation.

The usefulness of microplate tests for soil biochemical properties in assessing land condition, including that of remnant vegetation with native and exotic understorey was confirmed. This assay format was used with promising results to show the effects of several treatments simulating degradation and restoration on soils and vegetation, which
is of practical importance in management of remnant patches of native vegetation. In addition, it provided preliminary insights into the relationships among soil chemical and biochemical properties, which could contribute to the development of a high through-put, inexpensive and reliable tool for assessing and monitoring land condition and restoration efforts.
Table of contents

DECLARATION 3

ACKNOWLEDGEMENTS 5

ABSTRACT 7

TABLE OF CONTENTS 11

LIST OF TABLES 15

LIST OF FIGURES 17

LIST OF ABBREVIATIONS 19

CHAPTER 1. INTRODUCTION 21

1.1. Biodiversity, native vegetation remnants and plantations 22

1.2. Land use change and soil microbiota 25

1.3. Soil properties as indicators of changes in microbial communities 29
   1.3.1. Chemical soil properties 30
   1.3.2. Soil enzymatic activity 30
   1.3.3. Soil nitrogen mineralization potential 32
   1.3.4. Catabolic potential and community-level physiological profiling 32

1.4. Microplate tests for soil biochemical properties 35

1.5. Hypotheses and thesis outline 37

CHAPTER 2. METHODS 39

2.1. Study area 39

2.2. Laboratory protocols 42
   2.2.1. Soil collection 42
   2.2.2. Soil water content and maximal water-holding capacity 42
   2.2.3. Soil nutrients analysis 44
   2.2.4. Soil nitrogen mineralization potential with ammonium and nitrate determination in microplate format 44
   2.2.5. Catabolic potential 46
   2.2.6. Enzymatic activity assays 49

2.3. Statistical analyses 53
2.3.1. Data limitations 53
2.3.2. Variation partitioning 55
2.3.3. Constrained ordination 55
2.3.4. Data preparation and analytical procedures 56

CHAPTER 3. SOIL PHYSICAL PROPERTIES HAVE GREATER IMPACT ON SOIL CHEMISTRY THAN LAND CONDITION 59

3.1. Introduction 59

3.2. Materials and methods 60
3.2.1. Sites description and soil sampling 60
3.2.2. Laboratory methods and techniques 61
3.2.3. Statistical analysis 61

3.3. Results 64
3.3.1. Variation partitioning and db-RDA 64
3.3.2. Multiple Factor Analysis (MFA) 66

3.4. Discussion 70
3.4.1. Soil physical properties and the differences among land conditions 70
3.4.2. Differences in soil properties among land use types 74

3.5. Conclusions 77

CHAPTER 4. EFFECTS OF SHORT TERM NUTRIENT ENRICHMENT, WEED INVASION AND PHYSICAL DISTURBANCE ON VEGETATION AND BIOCHEMICAL SOIL PROPERTIES IN NATIVE WOODLAND REMNANTS 79

4.1. Introduction 79

4.2. Experimental design and procedures 81
4.2.1. Study sites 81
4.2.2. Experimental treatments 81
4.2.3. Data collection 82
4.2.4. Statistical analyses 84

4.3. Results 86
4.3.1. Differences in plant cover 86
4.3.2. The effects of experimental treatments on soil properties and vegetation – one factor 88
4.3.3. The effects of experimental treatment on soil properties and vegetation – three factors 91

4.4. Discussion 97
4.4.1. The effects of ryegrass and fertilization on native vegetation 97
4.4.2. Relationships between soil biochemical properties and ryegrass persistence 98
4.4.3. The effects of fertilizer on soil properties 99
4.4.4. The effects of clearing
4.4.5. Biochemical soil properties related to native vegetation regrowth after disturbance

4.5. Conclusions

CHAPTER 5. THE EFFECTS OF RESTORATION TREATMENTS ON SOIL BIOCHEMISTRY AND THE SURVIVAL AND GROWTH OF PLANTED SEEDLINGS

5.1. Introduction

5.2. Materials and methods
5.2.1. Experimental design and sampling procedures
5.2.2. Laboratory analyses
5.2.3. Statistical analyses

5.3. Results
5.3.1. The effects of treatment on soil biochemistry, exotic ground cover and seedlings growth and survival

5.4. Discussion
5.4.1. The effects of mulch
5.4.2. The effects of herbicides
5.4.3. The effects of plastic covering
5.4.4. Species-specific response to treatments

5.5. Conclusions

CHAPTER 6. FUNCTIONAL DIVERSITY AND RELATIONSHIPS AMONG SOIL PROPERTIES

6.1. Introduction

6.2. Materials and methods
6.2.1. Data collection and pre-treatment
6.2.2. Statistical analyses

6.3. Results
6.3.1. Functional diversity in soil from contrasting land conditions and treatments
6.3.2. Comparisons among experimental treatments in reference to land condition
6.3.3. Relationships among soil properties

6.4. Discussion
6.4.1. Functional diversity
6.4.2. Differences in soil properties between experimental treatments in the context of reference land conditions
6.4.3. Relationships between chemical and biochemical soil properties
6.5. Conclusions

CHAPTER 7. SYNTHESIS

7.1. Major findings of the study

7.2. Limitations of the study
   7.2.1. Data collection
   7.2.2. Laboratory analyses
   7.2.3. Interpretation of results

7.3. Possible directions for future research
   7.3.1. Biochemical indices of soil
   7.3.2. Investigation of restoration treatments
   7.3.3. Alternative methodologies for rapid and inexpensive large-scale soil analysis

BIBLIOGRAPHY:

APPENDIX 1. HERBICIDES AND HARVEST RESIDUE MULCH IN RESTORATION OF REMNANTS WITH EXOTIC UNDERSTOREY – PILOT TRIAL

8.1. Experimental treatments and data collection
   8.1.1. Laboratory analyses
   8.1.2. Statistical analyses

8.2. Results

8.3. Conclusions
List of tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1. Chemical soil characteristics selected for the study to be tested by Cumming Smith British Petroleum (CSBP) Ltd, Bibra Lake, Western Australia</td>
<td>44</td>
</tr>
<tr>
<td>2.2. Substrates used in the Protocol 1 for the microplate catabolic potential assay</td>
<td>47</td>
</tr>
<tr>
<td>2.3. Substrates used in Protocol 1 for the microplate catabolic potential assay</td>
<td>49</td>
</tr>
<tr>
<td>2.4. Substrates and reaction conditions of microplate assays for enzymatic activity in the soil</td>
<td>54</td>
</tr>
<tr>
<td>3.1. Subsets of variables for soil properties investigated in the study</td>
<td>63</td>
</tr>
<tr>
<td>3.2. Variation partitioning in the distance-based redundancy analysis model of the effects of land condition and location on soil physical, chemical and biochemical properties</td>
<td>64</td>
</tr>
<tr>
<td>3.3. Variation partitioning in the distance-based redundancy analysis model of the effects of land condition, location and soil physical properties (Structure) on soil chemical and biochemical properties</td>
<td>65</td>
</tr>
<tr>
<td>3.4. Significance of the canonical axes (CAPs) in the partial distance-based redundancy analysis model of the effects of land condition on soil physical, chemical and biochemical properties, after correction for the effect of location.</td>
<td>65</td>
</tr>
<tr>
<td>3.5. Proportions of the variation explained by the dimensions in the MFA model</td>
<td>66</td>
</tr>
<tr>
<td>3.6. Correlations among subsets of soil properties in multiple factor analysis model</td>
<td>68</td>
</tr>
<tr>
<td>4.1. Selected chemical properties of the soil in the study sites</td>
<td>83</td>
</tr>
<tr>
<td>4.2. Experimental treatments in the study investigating the degradation factors in remnants of native woodland within <em>E. globulus</em> plantations in the south west of Western Australia.</td>
<td>83</td>
</tr>
<tr>
<td>4.3. Native (NC) and exotic (EC) ground cover (%) in the study sites before and at 3 and 6 months after treatment application.</td>
<td>88</td>
</tr>
<tr>
<td>4.4. Effects of experimental treatments on soil properties and vegetation at 3 and 6 months after treatment detected by variation partitioning.</td>
<td>90</td>
</tr>
<tr>
<td>4.5. Significance of the canonical axes (CAPs) in the distance-based redundancy analysis of the effects of experimental treatments on soil properties and vegetation at 3 and 6 months after treatment application.</td>
<td>92</td>
</tr>
<tr>
<td>4.6. Effects of experimental treatments on soil properties and vegetation at 3 and 6 months after treatment detected by variation partitioning.</td>
<td>96</td>
</tr>
<tr>
<td>5.1. Selected chemical properties of the soil in the study sites.</td>
<td>107</td>
</tr>
</tbody>
</table>
5.2. Experimental treatments in the study investigating selected restoration practices in the remnants of native vegetation in *E. globulus* plantations in the south west of Western Australia.

5.3. Effects of experimental treatments on soil properties, exotic ground cover and planted seedlings survival and growth, detected by variation partitioning.

5.4. Significance of the canonical axes (CAPs) in the distance-based redundancy analysis of the effects of experimental treatments on soil properties, exotic groundcover and planted seedlings growth and survival.

6.1. Soil properties (abbreviated in parentheses) used in analyses in Chapter 6.

6.2. Forward selection of variables explaining variability in the biochemical soil properties in data set 5.

6.3. Variation in the soil biochemical properties explained by soil chemical properties as detected by variation partitioning in data set 5.

7.1. Soil properties most useful in discernment between land conditions and experimental treatments in this study.

8.1. Mean values (±SD) of selected chemical properties of the soil in the study sites.

8.2. Experimental treatments in the study investigating selected restoration practices in the remnants of native vegetation in *Eucalyptus globulus* plantations in the south west of Western Australia.

8.3. Effects of experimental treatments on soil properties, exotic ground cover and planted seedlings survival and growth, detected by variation partitioning.

8.4. Effects of experimental treatments on exotic groundcover and planted seedlings growth and survival at 11 months after treatment application, i.e. 10 months after planting, tested by variation partitioning.

8.5. Significance of the canonical axes (CAPs) in the distance-based redundancy analysis of the effects of experimental treatments on soil properties, exotic groundcover and planted seedlings survival and growth at 10 months after planting.
List of figures

Figure                                                                 Page
---                                                                     ---
2.1. Location and lithology of the sampling sites.                    40
2.2. Mean monthly maximum and minimum temperatures and rainfall averaged over last 70 years from the Albany Airport meteorological station. 41
2.3. Five land conditions common in the landscapes of the south west of Western Australia. 43
3.1. Differences in physical, chemical and biochemical soil properties between land conditions, detected by partial distance-based redundancy analysis. 67
3.2. Dimensions of the multiple factor analysis (MFA) assessed using the average eigenvalue rule (A) and broken stick model (B) and the correlations of the soil variables subsets and nominal factors to the first six MFA dimensions (C-E) 69
3.3. Differences among land conditions detected by multiple factor analysis (MFA). 71
4.1. Effect of the experimental treatments on native plant cover, exotic plant cover and number of native plant species at 3 and 6 months after treatment application. 87
4.2. Representative plots in site RG at three months after application of treatments mimicking degradation factors. 89
4.3. First two principal components of the analysis of soil properties before treatment application. 91
4.4. Effect of experimental treatments on soil properties and vegetation at 3 months after application, detected by partial distance-based redundancy analysis. 93
4.5. Effects of experimental treatments at 6 months after application on soil properties and vegetation detected by partial distance-based redundancy analysis. 94
4.6. Effects of the application of fertilizer on soil biochemical properties at 6 months after treatment application. 95
5.1. First two principal components of the analysis of soil properties before treatment application. 111
5.2. Effects of selected restoration treatments used in site S. 112
5.3. Effects of selected restoration treatments in site P. 113
5.4. Effects of rehabilitation treatments on the total ground cover of exotic grass and broadleaf plants. 115
5.5. Effect of rehabilitation treatments on the survival of the seedlings of the three native plans species. 117
5.6. Effects of various rehabilitation treatments on maximum and average height of the seedlings of the three native plant species. 118

5.7. Effects of the experimental treatments on soil properties at 11 months after treatment application, detected by partial distance-based redundancy analysis. 120

5.8. Effect of treatments on exotic ground cover and seedling growth and survival at (A, B) 3 and (C, D) 10 months after planting, detected by partial distance-based redundancy analysis. 123

6.1. Schematic diagram of the construction of data sets analysed in Chapter 6. 132

6.2. Differences in functional diversity calculated using soil chemical and biochemical properties among five land conditions in data set 1. 135

6.3. Differences in soil biochemical properties among treatments and land conditions detected for multiple factor analysis Dimensions 7 and 8. 137

6.4. Differences in soil biochemical properties among treatments and land conditions detected for multiple factor analysis Dimensions 10 and 12. 138

6.5. Correlation among soil properties and dimensions resulting from multiple factor analysis analysis of data set 5. 140

6.6. Grouping of soil biochemical properties as detected by redundancy analysis in data set 5. 141

6.7. Relationships between soil chemical and biochemical properties detected by redundancy analysis. 145

7.1. Schematic representation of the study. 153

8.1. First two principal components of the analysis of soil properties before treatment application. 197

8.2. Effects of selected restoration treatments at 4 months after application at site P. 198

8.3. Effects of restoration treatments on survival (A) and growth (B) of seedlings of six local native plant species in Study 1 at 11 months after planting. 200

8.4. Effects of planting and restoration treatments on exotic ground cover measures. 202

8.5. Effect of experimental treatments on exotic ground cover and seedlings survival and growth at 10 months after planting, detected by partial distance-based redundancy analysis. 203
List of abbreviations

AFS – Australian Forestry Standard
ANOVA – analysis of variance
APFL – Albany Plantation Forest Company
ARDRA – amplified ribosomal DNA restriction analysis
ARISA – automated ribosomal intergenic spacer analysis
CLPP – community-level physiological profile
Db-RDA – distance-based redundancy analysis
D/TGGE – denaturing/temperature gradient gel electrophoresis
DNA – deoxyribonucleic acid
FDA – fluorescein diacetate
FSC – Forest Stewardship Council
IN woodland – intact native woodland
MFA – multiple factor analysis
NGS – next generation sequencing
PerMANOVA - permutational multivariate analysis of variance
PCR-DGGE – polymerase chain reaction-denaturing gradient gel electrophoresis
PLFA – phospholipid fatty acid
QIIME – Quantitative Insights Into Microbial Ecology
RDA – redundancy analysis
SD – standard deviation
SWI – Shannon-Weaver Index
SWWA – south west of Western Australia
T-RFLP – terminal restriction fragment length polymorphism
UDE - native vegetation remnant with exotic understorey
UDN – native vegetation remnant with native understorey
WA – Western Australia
Chapter 1. Introduction

Soil microbial communities mediate processes essential to carbon (C) and nitrogen (N) cycling and influence ecosystem productivity through feedback mechanisms involving plant communities (Van der Putten et al. 2013; Bezemer et al. 2006, Verstraete 2004, Zak et al. 2003). Studies into soil microbial communities can enrich research focused on land use change, degradation and restoration, all of which are concerned with alterations of plant community. Biochemical indicators of microbial function in soils, such as catabolic potential profiles or enzymatic activity, constitute an integral part of such investigations.

This chapter presents the current state of knowledge on the use of soil biochemical properties as tools for monitoring restoration with the aim of using this to assess native vegetation remnants within Blue Gum (Eucalyptus globulus Labill.) plantations in the south west of Western Australia (SWWA). The chapter is divided into five sections: the first introduces the native vegetation remnants within plantations as the object of the study and outlines the issues associated with their management for biodiversity conservation in light of the forest certification schemes. The second describes the effects of land use change on soil microbial communities. The third provides examples of the use of soil chemical and biochemical properties as indicators of soil functioning in different land use management regimes, land use change and restoration. The fourth presents background information on the microplate format tests for soil biochemical properties and their application in monitoring land use change and rehabilitation efforts. The final section outlines the hypotheses of the current study, which aim to fill, at least partially, the knowledge gaps identified in the previous sections.
1.1. **Biodiversity, native vegetation remnants and plantations**

One of the major threats to biodiversity arises from the degradation brought about by anthropogenic land use change, resulting in loss of habitat for species (Lindenmeyer and Fisher 2006). This affects the majority of the ecosystems today (Ellis et al. 2010, Ellis and Ramankutty 2008). Over half of the terrestrial species of plants and animals inhabit forest ecosystems (Millenium Ecosystem Assessment 2005), and anthropogenic impacts such as deforestation cause large effects on biodiversity. The results of anthropogenic intervention into ecosystems, including forests, are especially visible in the biodiversity hotspots, such as the SWWA. European settlement in Australia brought a landscape-scale conversion of native vegetation to agriculture (Yates and Hobbs 1997) and as a result, over two-thirds of the original vegetation has been removed over the course of nearly 200 years in SWWA (Beard and Sprenger 1984). Since the early 2000’s, no native eucalyptus open forests, and only 50-70% of other types of the native forest and woodlands, dominated by jarrah (*Eucalyptus marginata*) and marri (*Corymbia calophylla*), remain in that region (Australian Natural Resources Atlas, 2000-2002). As a result of such extensive clearing, many plant species have suffered extinction or severe decline in population (Hobbs and Yates 2000). The practice of leaving patches of native woodlands and forests, growing near rocky outcrops or on poorer, gravelly or shallow soils has created a mosaic of cropland, pastureland and native woodland remnants of size varying from 1 to tens of hectares. The majority of the smaller remnants, surrounded by pastureland, were commonly used by farmers as woodlots and livestock shelters, with severe consequences for the native vegetation in these patches (Hobbs and Yates 2000).

Over recent decades, the plantation industry has afforested over 300,000 ha of agricultural land with *E. globulus* (Gavran and Parsons 2010), a species native to eastern Australia. Several studies have reported positive effects of the establishment of tree plantations on animal and plant biodiversity in Australia (for example Hobbs et al.)
2003, Lindenmeyer and Hobbs 2004). The afforestation of pastureland with *E. globulus* is providing an opportunity for the rehabilitation of land that has been cleared for agriculture. Establishing single-age, single-species plantations, which enclose native vegetation remnants in a matrix of exotic forest, can buffer the remnants against influences of the neighbouring non-forested areas, decreasing the edge effect on native biodiversity (Wright *et al.* 2010, Denyer *et al.* 2006). The management of native vegetation patches remaining in the areas bought by or leased to the plantation companies becomes the responsibility of the industry. Recently, the advent of forest product certification provided an additional incentive for plantation companies to explore the options for management of the native vegetation within their estates.

The considerable conservation value of forest or woodland remnants from a biodiversity perspective has been recognized in the Australian Forestry Standard (AFS) and Forest Stewardship Council (FSC). Compliance with the policies issued by these authorities, outlining broad guidelines for the forest management, is the basis of certification, and is attractive to the plantation industry due to increased market value of the certified products. The standard for compliance outlined in the certification guidelines include management for biological diversity, which is defined in the FSC as "the variability among living organisms from all sources including, *inter alia*, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are a part; this includes diversity within species, between species and of ecosystems" (FSC Principles and Criteria for Forest Stewardship 1996, p.10); and by AFS as "the diversity of plants, animals and other living organisms in all their forms and levels of organization, (which) includes the diversity of genes, species and ecosystems, as well as the evolutionary and functional processes that link them" (AS 4708—2007 p.12). Even small remnants of native vegetation of less than 2 ha can serve as repositories of native plant seeds, provide habitats for native fauna and act as "stepping stones" for the movement of some of the native species across landscapes (Lindenmayer and Fisher 2006, Hobbs *et al.* 2003, Yates and Hobbs 1997). The age of the remnants is another important
consideration, as the older trees provide nesting resources for many local species of small mammals and reptiles in tree hollows and in piles of coarse woody debris (Lindenmayer and Fisher 2006).

Few studies have focused on the biodiversity of remnants in *E. globulus* plantations in the SWWA and on the impact of the land use changes in these landscapes. The information on the monitoring of the condition and on the management of these remnants is sourced from much more abundant research on the remnants within pastures or cropland in eastern Australia (Davidson *et al.* 2008, Close *et al.* 2007, Spooner *et al.* 2002) and in the wheatbelt area of WA (Prober and Smith 2009). Such studies revealed that native vegetation remnants are exposed to a number of anthropogenic pressures, such as grazing by livestock, excessive soil nutrients supplied by artificial fertilization of the nearby fields and livestock camping in the remnants (Duncan *et al.* 2008), and soil compaction (Davidson *et al.* 2007, Spooner *et al.* 2002). The excess of nutrients is presumably directed into short-term cycling in exotic, herbaceous annuals, which out-compete native woody perennials in the understorey. This can negatively impact remnants’ regeneration by decreasing native species recruitment and young plants’ survival (Close *et al.* 2008).

Exclusion of the grazers was found to be a very important step in improving the condition of remnant vegetation (Close *et al.* 2008, Spooner *et al.* 2002, Yates *et al.* 2000b); however, it is rarely enough to restore remnants to their pristine state, or even to ameliorate their degradation, as the persistence of weeds, facilitated by nutrient-enriched soils, contributes to a cycle of decline (Archibald *et al.* 2011). In addition, research from other ecosystems indicate that fragmentation of forests induces changes in the vegetation composition, which are exacerbated by the time from isolation, and include the loss of plant species characteristic of mature forest, in favour of the early successional and ruderal species (Zhu *et al.* 2010). Further, losses of large, mature trees from native forest fragments embedded within crop monocultures such as sugarcane plantations, reported in Brazil, were related to the age of the remnants (Oliveira *et al.*
2008, Santos et al. 2008). This indicates that the longer the remnants remained unmanaged, i.e. unprotected, the smaller the chance of recruitment from the mature trees seed-bank and the bigger the risk of the remnants' eventual disappearance (Oliveira et al. 2008, Santos et al. 2008). It seems that the benefits of active and immediate remnant restoration are substantial; however, there are still gaps in the knowledge regarding how to restore them effectively.

Even though soils can be strongly impacted by land use changes, very little is known about the impacts of management practices and processes leading to degradation or restoration on soils. This is true in general, and is particularly true for soil processes that occur in fragmented landscapes of Western Australia. Soil microbes play pivotal role in many nutrient cycling processes and thus can have a profound impact on plant communities. Soil microorganisms are thought to have significant effects on plant productivity, as they are responsible for up to 100% of decomposition of organic matter, up to 20% of N fixation and up to 90% of plant P acquisition (for review see Van der Heijden et al. 2008). The diversity of plant community can be influenced by soil microbes, for example in grassland ecosystems, where arbuscular mycorrhizal fungi were shown to have the capacity to increase or decrease it (Van der Heijden et al. 2008). The effects of soil microbiota on plant communities include also shifts in composition, that can be due for example to the activity of soil-borne pathogens, or to facilitation of exotic plant invasion as a result of symbiotic interactions with native soil microbes (for review see Van der Putten et al. 2007). There is a very limited number of studies that explore the functioning of soil microbes in native vegetation remnants or their response to the rehabilitation treatments or degrading factors (for example Yates et al. 2000a, b).

1.2. Land use change and soil microbiota

Different characteristics of soil microbial communities have been used in attempts to investigate changes in soil microbiota (for review see Nannipieri et al 2003). Microbial
biomass, i.e. weight of live microorganisms in a unit of soil volume or surface area, could be estimated for example by plate or direct counts; measurement of C, N and P in the chloroform fumigation and extraction procedure; phospholipid fatty acid (PLFA) analysis or glucose-induced respiration. Microbial activity, referring to the vast range of biological activities that soil microorganisms perform, is often measured by biochemical methods, as rates of whole metabolic process, such as catabolism in CLPP, or hydrolytic activity of a range of enzymes as in FDA hydrolysis assay.

Studies of the composition of microbial communities, such as determination of the constituents of the microbial population or the number (richness) and abundance (evenness) of bacterial and fungal species in a given habitat can be carried out for example by investigating genetic diversity using molecular methods, such as nucleic acid fingerprinting: denaturing/temperature gradient gel electrophoresis (D/TGGE), amplified ribosomal RNA restriction analysis (ARDRA), automated rRNA intergenic spacer analysis (ARISA), terminal restriction fragment length polymorphism(T-RFLP). More recently, a direct identification of the microorganisms present in a soil sample and determination of their abundance using high through put next generation sequencing (NGS) has become available. While still too expensive for routine testing, with decreasing costs this methodology will be more and more accessible, possibly leading to major advances in soil microbiology and microbial ecology. Of the non-genetic approaches, both PLFA analysis and functional diversity measurements in CLPP were successfully used to determine changes in microbial communities (Nannipieri et al. 2003).

Land use change can promote formation of distinct microbial assemblages in the soil by altering vegetation cover, which affects microbial activity in soils by modulating temperature, moisture relations and nutrient input, for example, via litter quality and quantity and root exudation (Perez-Bejarano et al. 2010, Ernst 2004).

The effects of vegetation cover on soil microbial communities and their biochemical indices have been observed using different methods in a variety of locations, vegetation types and climates. Different microbial communities have been
found in soils under pasture, as compared to pine plantation (Macdonald et al. 2009, Kasel et al. 2008, Stevenson et al. 2004), or soils under different crop types (Bossio et al. 2005, Nsabimana et al. 2004) and soils subjected to different restoration practices (for example, in rehabilitated forest following bauxite mining: Cookson et al. 2008a, Lalor et al. 2007). In most of these studies, the community-level physiological profiling (CLPP) approach was used; however, other methods for this type of investigation have been used successfully worldwide. For example, Habekost et al. (2008) revealed significant changes in soil microbial communities four years after a change from arable to grassland using PLFA. T-RFLP method allowed Kasel et al. (2008) to show a distinction between fungal communities in soils of four different land uses in eastern Australia: native eucalypt forest, pasture, and Pinus radiata and E. globulus plantations. Bossio et al. (2005) used PLFA, BIOLOG™, polymerase chain reaction – denaturing gradient del electrophoresis (PCR-DGGE) and analyses of soil enzymatic activity to reveal differences between land uses (forest, woodlot, agricultural land), soil types and management practices. The BIOLOG™ assay has also been used to reveal differences in soil microbial communities in pure and mixed stands of conifers and red alder (Selmants et al. 2005).

The effect of a single dominant plant species on soil has been investigated in detail with respect to soil enzymatic activity in Western Australia (Marschner et al. 2005, Grierson and Adams 2000) and elsewhere (e.g. Chacon et al. 2009, Allison et al. 2006, Selmants et al. 2005). In particular, Marschner et al. (2005) provided evidence, using enzymatic activity tests and PCR-DGGE, that three closely related plant species in the same genus (Banksia) can harbour distinctively different microbial communities. Dinesh et al. (2004) reported changes in the activity of a suite of soil enzymes associated with a change from native forests to tree plantations in a wet tropical climate. Paz-Ferreiro et al. (2009) reported differences in soil enzymatic activity of soil from undisturbed mature and improved or re-sown grasslands.
Clearing and removal of vegetation can cause decreases in microbial biomass and enzymatic activity (Caldwell et al. 1999) and increase N-mineralization in temperate forests soils (Morris and Boerner 1998a, b). As soil properties are influenced by the plant species growing in the area (Marschner et al. 2005), invasion of exotic plant species can dramatically alter soil biochemical properties and nutrient cycling (Allison et al. 2006).

Nutrient enrichment of soil has profound consequences for microbial life in soils (Chaparro et al. 2012), for example, by increasing microbial biomass (Degens et al. 2000, Degens and Harris 1997) or causing shifts in the composition of microbial populations (Peacock et al. 2001). Accordingly, biochemical indices of microbial activity in the soil can be influenced by changes in soil nutrient status. For example, long-term N fertilization increased the activity of β-glucosidase and acid phosphomonoesterase, and decreased that of urease in tallgrass prairie soils (Ajwa et al. 1999).

Moreover, soil microbial communities can be influenced by non-biotic factors such as site characteristics, physical and chemical soil properties. Kasel et al. (2008) reported differences in soil fungal communities between three different locations. In another study, soil type was found to have more influence on soil microbial communities than time, field management, or spatial variation (Bossio et al. 2005). However, enzymatic activity and BIOLOG™ analyses “were less specific with regard to soil type than the 16S rRNA gene DGGE and PLFA approaches” (Bossio et al. 2005), which highlights the importance of the choice of appropriate methodology in such investigations.

Responsiveness of soil microbial communities to such a wide range of influences is important in the case of remnant vegetation, as many of the drivers of changes in microbial communities are multiplied. For example, grazing by livestock causes not only changes in vegetation structure and composition and soil compaction, but also soil nutrient enrichment, especially P and N, which encourages the invasion and persistence of exotic plants and concomitant changes in soil microclimate (Yates et al. 2000a, 2000b,
Yates and Hobbs 1997). When greater amounts of nitrate, total N and P were present in the soil of remnant vegetation surrounded by pasture in Tasmania, poorer tree health and survival was in evidence (Close et al. 2008). Using biochemical soil properties as indicators of microbial activity may be a useful tool in monitoring the condition of remnant vegetation and could contribute information necessary for their optimal management for biodiversity conservation.

1.3. **Soil properties as indicators of changes in microbial communities**

Several indicators of healthy soil function have been proposed (e.g. Lagomarsino et al. 2009, Benintende et al. 2008, Dale et al. 2005, Schloter et al. 2003, Pankhurst et al. 1995); however, a general indicator for use in different ecosystems has not yet been agreed upon despite many efforts (Velasquez et al. 2007, Nannipieri et al. 2003, Filip 2002). Individual soil biochemical properties, such as activity of enzymes or N-mineralization potential, have been used to determine the state and functionality of soils, and were found to be more sensitive than soil chemical properties in detecting the effects of agricultural land management and land use change (Lagomarsino et al. 2009, Zornoza et al. 2009, Benintende et al. 2008, Chapman et al. 2003). Specifically, enzymatic activity has been recognized as an important indicator of soil function and microbial status (Caldwell 2005, Nannipieri et al. 2003, Bandick and Dick 1999).

A widely used indicator of microbial activity is soil catabolic potential, or substrate-induced respiration, which can provide physiological profiles of the microbial metabolism at a community level (Campbell et al. 2003, Degens and Harris 1997). Catabolic potential has been shown to respond to change in land use, disturbance, cropping, and soil organic carbon pools (Stevenson et al. 2004, Degens et al. 2001, Griffiths et al. 2001, Degens et al. 2000). Soil N mineralization potential is another simple indicator of the capacity of soil to supply inorganic N, an important nutrient for plant productivity and health (Doran and Parkin 1994). Nitrogen mineralization potential of soil
was reported to change with different cropping regimes (Benintende et al. 2008) and with heavy clearing (Morris and Boerner 1998a).

The biochemical properties of soil are also influenced by changes in land management. For example, thinning and fuel-reduction burning altered soil respiration, enzymatic activity and microbial physiological capabilities in temperate pine (Boyle et al. 2005) and mixed-oak forest (Giai and Boerner 2007) in the USA. Changes in enzymatic activity of soil from tallgrass prairie following long-term fertilization and burning were measured even when total N and C content of soil remained unchanged (Ajwa et al. 1999). Moreover, increases in the activity of a range of soil enzymes such as dehydrogenase and hydrolases (β-glucosidase, urease and acid phosphomonoesterase) were measured after revegetation of post-mining soils with native tree species in the subtropical climate of north-east of Cuba (Izquierdo et al. 2005).

1.3.1. Chemical soil properties

The activity of soil microbes is related to, and modulated by, soil chemical properties, including moisture, pH and the availability of nutrients (Cookson et al. 2008b). Soil pH was thought to be the biggest driver of soil microbial function and genetic diversity in a range of Australian soils (Wakelin et al. 2008). On the other hand, Cookson et al. (2008b) reported soil organic C and inorganic N content as the most important factors determining catabolic potential of the soil in restoration of mining sites. In addition, soil moisture has been found to influence soil microbial communities more than soil C and N (Singh et al. 2009). Bunemann et al. (2006) provide an extensive review of the effects of a wide range of soil amendments, including organic and inorganic fertilizers and pesticides on soil microbiota.

1.3.2. Soil enzymatic activity

The turnover of nutrients from detritus and accumulated soil organic matter requires the activity of extracellular enzymes (Caldwell 2005) that can be produced and
secreted by living organisms, as well as those which “leak” from dead organic matter (Dakora and Phillips 2002, Verstraete and Mertens 2004). Enzymes contribute to the decomposition of many macromolecules present in the soil, including lignin and cellulose, starch, lipids, proteins and chitin (Wallenstein and Weintraub 2008). This process ensures that the cycling of the major nutrients occurs. With this in mind, soil enzymatic activity has been recognized as an important indicator of soil function and microbial status (Caldwell 2005, Bandick and Dick 1999).

The activity of soil enzymes can be influenced by a variety of factors (Nannipieri et al. 2012, Gianfreda and Ruggiero 2006), including season (Hoyle and Murphy 2006, Grierson and Adams 2000), plant community composition (Selmants et al. 2004), climate change, particularly increases in CO₂ (Drissner et al. 1999), soil disturbance, such as compaction (Kissling et al. 2009), drought (Sardans and Penuelas 2005), addition of fertilizer and fire (Boyle et al. 2005, DeForest et al. 2004, Boerner et al. 2008, Ajwa et al. 1999), crop rotation and soil amendments (Bandick and Dick 1999), and tillage (Kandeler et al. 1999). The majority of methods available for measuring soil enzymatic activity involve the use of natural or artificial substrates which are converted to coloured or fluorescent products by soil enzymes (for review see for example Caldwell 2005). Claims of usefulness of enzymatic activity as an indicator of soil quality has been made for example in cases of β-glucosidase (Stott et al. 2010) and fluorescein diacetate hydrolysis (Aseir and Tarafdar 2006) assays; however, conclusions from such studies may have to be interpreted with caution due to a variety of limitations inherent in the methodology (for review see Nannipieri et al. 2012, Nannipieri et al. 2003). Most of the methods allow estimation of the potential enzymatic activity under unrealistically optimal conditions and often using artificial substrates (Nannipieri et al. 2003). Wallenstein and Weintraub (2008) suggest that the accuracy and relevance of results obtained using enzyme assays are questionable. However, due to the ease of the methods involved and tradition, they are still a common approach to measuring soil microbial activity. Molecular approaches such as proteomics have also been reported (Renella et al. 2013, Wilmes
and Bond 2006, Schulze 2004), although their use is not as routine (for review see Myrold and Nannipieri 2013; Bastida et al. 2009).

### 1.3.3. Soil nitrogen mineralization potential

Nitrogen mineralization is a series of microbe-mediated processes, involved in the conversion of organic N into inorganic forms which are available to plants. This process has implications for crop productivity and the use of artificial N fertilizer, and is an important measure of agricultural productivity. A new paradigm in the understanding of the N-mineralization processes has been developed in late 1990s, placing the depolymerisation of organic matter, rather than N mineralization process, at a critical point of N cycling in soils (reviewed in Schimel and Bennett 2004). Soil N-mineralization has been proposed as a possible indicator of disturbance in forest soils, as clear-felling or heavy thinning increased the potential N-mineralization in oak-dominated stands studied by Morris and Boerner (1999a); however, a study by Barg and Edmonds (1999) found no effects of clearing on N-mineralization. Nevertheless, the usefulness of measuring this process for the purpose of monitoring soil condition was suggested by Benintende et al. (2008), who reported that soil N-mineralization potential was more sensitive to the effects of crop rotation than traditional measures of organic C and total N content. Biological and chemical methods for assessing N-mineralization in soils have been proposed and optimized; Cordovil et al. (2007) provides a comparison of the chemical methods.

### 1.3.4. Catabolic potential and community-level physiological profiling

Anderson and Domsh (1978) introduced a method of measuring soil metabolic activity by detecting CO$_2$ generated by soil incubated in airtight containers. The method has since been modified and widely used for many purposes. Glucose-induced respiration has become a functional alternative to chloroform fumigation for estimating
microbial biomass (Sparling and Zhu 1993, Anderson and Domsh 1978). The selective inhibition of microbes using antibiotics has also been used to distinguish between fungal and bacterial components of soil functioning (Ananyeva et al. 2006, Bailey et al. 2003).

The generation of CO$_2$ by soil in response to amendment with different C and N sources has led to the development of assays for microbial functional diversity, analogous to the BIOLOG™ plate tests (Garland and Mills 1991), the most common of which is catabolic potential (Degens and Harris 1997) and Community-Level Physiological Profiling (CLPP) (Campbell et al. 2003). For these assays, a carbon source is added to the soil and the CO$_2$ generated as a result of this amendment is measured. Using the whole soil approach overcomes some of the shortcomings of the otherwise useful BIOLOG™ method, such as dependence on the aqueous extraction and culture of microbes, which induces changes in microbial composition of the extracts, as well as the concentration and relevance of the substrates used (Chapman et al. 2007, Campbell et al. 2003, Konopka et al. 1998).

Carbon sources that have been investigated as substrates in the CLPP include carboxylic acids, amino acids, alcohols, carbohydrates, amines, amides, aromatic chemicals and polymers (Campbell et al. 2003, Degens and Harris 1997). Many of these are root exudates (Badri and Vivanco 2009, Campbell et al. 1997) which can defend plants against pathogens, for example by disruption of pathogen signalling, direct killing of the pathogen or attracting symbionts or helping in nutrient acquisition (Dakora and Phillips 2002). In response to nutrient shortages, plants release specific root exudates, which are believed to assist in nutrient acquisition (Badri and Vivanco 2009, Dakora and Phillips 2002). Since the soil microbial community has developed to utilise available food sources, the injection of plant “starvation signals” in the form of nutrient-solubilizing exudates can change the microbial community which adapts to use them as food source. This new specialised community can be detected via increased respiration in response to the “starvation signal”, added in excess in the CLPP assay, as, for example, in the enrichment-type experiment of Degens (1998). The carbon can be sourced from a
diversity of plant-, microbe- and man-made molecules, as in the case of the fuel-contaminated soils reported by Kaufmann et al. (2003). This suggests that many different substrates could be used for physiological profiling assays, which makes the method very flexible and promising for a wide spectrum of applications (Chapman et al. 2007). However, rhizosphere-derived substrates have shown a tendency to distinguish microbial communities better than a wide range of general substrates (Campbell et al. 1997).

Previous research suggests that soil catabolic potential tested using a series of organic substrates can be altered by change in land use, disturbance, cropping, or soil organic carbon pools (Degens et al. 2001, Griffiths et al. 2001, Degens et al. 2000, Degens and Vojvodic’-Vukovic’ 1999). In a study from New Zealand, Stevenson et al. (2004) investigated microbial communities using a substrate-induced respiration method with soils from pasture, native forest and a pine plantation. They reported a clear distinction between these land uses, not only between pasture and forest, but also between native and exotic forest, with only nine substrates. Differences have been detected among soil microbial communities using this method in a study of the impact of the successional changes in vegetation on soil (Schipper et al. 2001).

Several methodological factors influence the outcome of the CLPP tests. The amount of C added to soil samples in an assay is of considerable importance, as carbon concentration-induced shifts in microbial communities have been found (e.g. Griffiths et al. 1999). The length of incubation is also argued to have an impact (Campbell et al. 2003, Degens 1998). The form in which the substrate is added to soil (e.g. the amount of water used for substrate delivery) can affect the results of the assay by influencing the dispersion of the substrate (Degens 1998), and by changing the environment in which respiration occurs (West and Sparling 1986). Lalor et al. (2007) used the same substrates as Stevenson et al. (2004) to compare directly the “soil suspension” (Degens and Harris 1997) with the “whole soil” approach (Campbell et al. 2003) to the catabolic potential testing and concluded that the latter was more useful for discerning between
treatments in a post-mining rehabilitation trial in the jarrah forest in WA (Lalor et al. 2007).

1.4. **Microplate tests for soil biochemical properties**

Soil characteristics such as chemical composition and biological and physical properties are interconnected and influence each other extensively (Nannipieri et al. 2003). Untangling such complex relationships to obtain a clearer picture of the dependencies and discover their causative factors represents a significant challenge. This is further exacerbated by the difficulty in obtaining systematic, landscape-scale data for many soil biochemical attributes. The natural variability of soils represents a serious obstacle for conducting this type of research (for example, Franklin and Mills 2007) and necessitates extensive sampling and multiple replicates or tests per sample, and therefore incurs higher costs in reagents and analysis time. Miniaturization of the tests to the microplate format is one of the ways to solve these problems.

The microplate approach for testing biochemical properties has been applied to a range of environmental samples including soil, water and sediments. Reports of successful measurement of biochemical properties in less than 0.5 g samples of soil are well documented. For example, Lai et al. (1998) have measured soil phosphomonoesterase and urease activity in 200 mg of soil. Mora et al. (2005) reported on the activity of a series of enzymes: acid and alkaline phosphomonoesterase, laccase, xylanase, amylase, cellulase, β-xylosidase, β-glucosidase and α-glucosidase in 100 mg samples. Microplate assays for enzymatic activity in soil extracts and diluted suspensions have also been successfully performed for litter-decomposing enzymes including peroxidase, phenol oxidase (Sayia-Cork et al. 2002, Johnsen and Jacobsen 2000), urease, acidic phosphomonoesterase, β-1,4-glucosidase and L-leucine aminopeptidase (Sayia-Cork et al. 2002).
For catabolic potential, the MicroResp™ approach, based on small quantities (100-300 mg) of soil in wells of a 96-well microtiter plate has been used (e.g. Campbell et al. 2003, Lalor et al. 2007). Garland et al. (2003) proposed another microscale approach to catabolic potential testing, later optimized by Zabaloy et al. (2008) using fluorescence-based detection of substrate-induced oxygen depletion in 150 µl of soil suspension. Additionally, measurement of nitrogenous species (nitrate, ammonium, total N) from soil extracts in microplate format has been shown to give results as reliable as those obtained using conventional methods (Sims et al. 1995).

Although there are issues of considerable intra- and inter-assay variation, as well as sample representativeness when dealing with small soil samples, microscale assays offer several advantages to the user. These include increased throughput and simplification of the testing process, with the possibility of automation, allowing for more samples in higher replication to be tested, while using smaller quantities of reagents and generating less waste (Chapman et al. 2007). The choice of sample preparation method, namely whole soil or soil suspension or extract, for assays of soil biochemical properties is important from the point of view of practicality and reproducibility of the results. Sample disturbance influences the results of soil oxygen usage assays (Zabaloy et al. 2008); however, successful use of soil suspension has been documented. For example, a soil slurry at 1:2.5 w/v, equivalent to 400 mg soil, was used for a series of standard para-nitrophenol (pNP)-based tests by Caldwell et al. (1999). High dilutions of soil, used in the fluorescence-based activity assays for litter-decomposing enzymes can increase homogenization which results in good assay precision and is well suited for use with highly sensitive fluorescent substrates (Sayia-Cork et al. 2002).

Small-scale approaches such as these are already widely used in restoration research. For example, the catabolic potential method (Campbell et al. 2003, Degens and Harris 1997) has been successfully used to monitor and assess the effects of management practices and rehabilitation (e.g. Cookson et al. 2008, Lalor et al. 2007, Hoyle and Murphy 2006).
1.5. **Hypotheses and thesis outline**

The first aim of this research was to investigate the potential of soil chemical and biochemical properties as indicators of soil status for the specific purpose of assessing degradation factors and restoration treatments in native vegetation remnants enclosed in *E. globulus* plantations in SWWA. The second aim was to determine which degradation factors and restoration treatments were most influential on native and exotic vegetation and on soil biochemical properties. This information will be valuable to inform plantation managers with remnant forests in estates which are under certification schemes. The third aim was to investigate patterns in the relationships among soil properties. This would be valuable in detecting predictors of the processes involved in nutrient cycling, degradation and restoration and may prove useful in developing indicators of land condition, disturbance severity and the efficiency of restoration practices.

The hypotheses of this research were:

1. Differences in ecologically relevant soil biochemical properties that can be detected using high throughput, microplate assays, allow distinction among land conditions in the SWWA, including those common to remnant vegetation in *E. globulus* plantations.

2. Nutrient enrichment is more influential in altering vegetation cover and soil biochemical properties than other degradation factors.

3. Selected restoration practices increase the success of the planted native seedlings and alter biochemical properties in the soils of the remnant native vegetation.

4. An index based on microplate tests for soil biochemical properties is indicative of land condition.
To test these hypotheses, a series of field experiments were done. These involved testing a range of soil attributes according to the protocols described in Chapter 2, including (a) chemical soil properties, routinely used for soil characterization in agriculture, and a selection of biochemical soil properties; (b) the activity of several enzymes involved in cycling of the major nutrients such as C, N, P and S; (c) nitrogen mineralization potential; and (d) catabolic potential assays.

An exploratory sampling study to investigate the differences in soil biochemical properties among five land conditions common in SWWA was completed as a “proof-of-concept” trial (Chapter 3). Subsequently, an experiment to investigate the effects of disturbance on soil biochemical properties and features of the native vegetation was conducted (Chapter 4). Then, the effects of several potential restoration treatments on soil biochemical properties and native seedling success were investigated in a pilot trial (Appendix I) to pre-screen the treatments and plant species to be used in a subsequent short-term experiment (Chapter 5). In addition, the links between soil functional diversity, as assessed using microplate tests for soil biochemical properties, and land condition were explored and an investigation of the relationships between soil properties was conducted using the combined data from all of the experiments (Chapter 6). A discussion of the major findings of the study in the context of the available literature, their implications and possible directions for future research are outlined in the final chapter (Chapter 7).
Chapter 2. Methods

2.1. Study area

The study area was located between Mt. Barker and Albany, approximately 450 km south east of Perth, Western Australia (Figure 2.1). The area has a mild, Mediterranean-type climate, with mean minimum temperatures ranging from 7.5 to 14.4 °C in winter and mean maximum from 15.8 to 24.9 °C in summer (Figure 2.2 a; Bureau of Meteorology 2011). The majority of the rain falls between April and October, averaging around 800 mm per year (Figure 2.2 b; Bureau of Meteorology 2011).

The soils in the area are sandy and loamy tenosols and podosols (Australian Soil Classification 2014). Lithology mostly comprises migmatite and gneiss of protoerozoic or uncertain age and tertiary sands (Fig. 2.1, Department of Water 2012). Lateritic boulders on the soil surface were present at one site (Mu). Historically, the soils supported temperate eucalypt woodland dominated by jarrah (E. marginata) and marri (Corymbia calophylla) (Archibald et al. 2011, Yates and Hobbs 1997). Examples of typical vegetation cover for the various land use types in the area are presented in Fig. 2.3. Managed pastures supporting cattle and sheep grazing (Fig. 2.3 b), and E. globulus plantation stands in the first and second rotation (Fig 2.3 e) dominate the landscape in the area.
Figure 2.1. Location and lithology of the sampling sites. Letters code for site names. Colours represent lithology: - gneiss, migmatite of uncertain age; - gneiss, migmatite - proterozoic; - sands - tertiary; - granitoid - proterozoic; - undifferentiated extensive sedimentary of uncertain age. Letters stand for abbreviations of the names of study sites. Dashed line represents pooling of the soil samples into four locations for the experiment described in Chapter 3. Adapted from Hydrogeological Atlas: http://www.water.wa.gov.au/idelve/hydroatlas/; accessed 26/10/2012.
Figure 2.2. Mean monthly maximum and minimum temperatures and rainfall averaged over the last 70 years from the Albany Airport meteorological station: 34.94°S 117.82°E located approximately 20 km south of the study sites (Bureau of Meteorology 2011).
2.2. Laboratory protocols

2.2.1. Soil collection

Samples of topsoil were collected from field sites using 5 cm corers at a depth of 0 to 5 cm. Care was taken not to include the organic layer (O horizon) and this layer was scraped away prior to inserting the corer, to eliminate bias due to differences in vegetation cover, and thus litter depth and composition, between sites. At least eight cores were taken at each site and bulked to form a composite sample. Soil samples were sealed in plastic bags to avoid moisture loss and transported to the laboratory at ambient temperature. Soil samples selected for biochemical analysis were hand-mixed and passed through a 2 mm sieve. Soil samples were analysed as described below within three days of collection.

2.2.2. Soil water content and maximal water-holding capacity

Soil water content and maximal water-holding capacity (maxWHC) were measured using a standard procedure adapted from Buurman et al. (1996). Briefly, field moist soil samples (20 to 40 g) were placed in pre-weighed 100 ml screw-cap containers with holes in the bottom and lined with filter paper (Whatman #1). Samples were weighed, caps were removed and containers were put in trays and slowly immersed in distilled water for overnight soaking at room temperature. After soaking, the containers were capped to prevent evaporation and transferred to sand-filled trays for draining until constant weight was reached. Samples were reweighed before removing the lids and drying in an oven at 105 °C for 12 h. The containers were cooled to room temperature in a desiccator and reweighed. Field moisture and maxWHC were calculated according to the equations 2.1 and 2.2, respectively.

\[
Moisture(\%) = \frac{\text{field moist weight (g)} - \text{dry weight (g)}}{\text{field moist weight (g)}} \times 100\% \quad \text{(Equation 2.1)}
\]

\[
MaxWHC(\%) = \frac{\text{wet weight (g)} - \text{dry weight (g)}}{\text{dry weight (g)}} \times 100\% \quad \text{(Equation 2.2)}
\]
Figure 2.3. Five land conditions common in the landscapes of the south west of Western Australia: (A) intact native woodland – IN woodland; (B) managed pasture; (C) remnant of native vegetation within *E. globulus* plantation, with moderately disturbed (native) understorey - UDN; (D) remnant of native vegetation within *E. globulus* plantation, with strongly disturbed (exotic) understorey - UDE; (E) mature *E. globulus* plantation. Photos in (A) and (C) courtesy of Dr R. Archibald.
Additionally, moisture content as a percentage of maximum water holding capacity was calculated according to Equation 2.3. This value was used to describe soil moisture content relative to maximum potential saturation which is relevant to soil biological activity.

\[
M_{\text{max WHC}}(\%) = \frac{\text{Moisture}(\%)}{M_{\text{ax WHC}}(\%)} * 100\% 
\]  
(Equation 2.3)

2.2.3. Soil nutrients analysis

Soil samples were air dried for >72 h at 35 °C then assayed by a commercial laboratory (Cumming Smith British Petroleum (CSBP) Ltd, Bibra Lake, WA) for total organic C, total N, ammonium-N, nitrate-N, conductivity, pH in CaCl₂, total sulphur and exchangeable phosphorus and potassium (Table 2.1).

Table 2.1. Chemical soil characteristics selected for the study to be tested by Cumming Smith British Petroleum (CSBP) Ltd, Bibra Lake, Western Australia. Adapted from Archibald (2006).

<table>
<thead>
<tr>
<th>Soil characteristic (unit)/Abbreviation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium-N (mg kg⁻¹)/A</td>
<td>Searle (1984)</td>
</tr>
<tr>
<td>Nitrate-N (mg kg⁻¹)/NitN</td>
<td>Searle (1984)</td>
</tr>
<tr>
<td>Exchangeable phosphorus - Colwell (mg kg⁻¹)/P</td>
<td>Rayment and Higginson (1992)</td>
</tr>
<tr>
<td>Exchangeable pPotassium - Colwell (mg kg⁻¹)/K</td>
<td>Rayment and Higginson (1992)</td>
</tr>
<tr>
<td>Sulphur (mg kg⁻¹)/S</td>
<td>Blair et al. (1991)</td>
</tr>
<tr>
<td>Organic Carbon (%)/OrgC</td>
<td>Walkley and Black (1934)</td>
</tr>
<tr>
<td>Total Nitrogen (%)/TotN</td>
<td>Rayment and Lyons (2010)</td>
</tr>
<tr>
<td>Conductivity (dS m⁻¹)/Cond</td>
<td>Rayment and Higginson (1992)</td>
</tr>
<tr>
<td>pH in CaCl₂ /pH</td>
<td>Rayment and Higginson (1992)</td>
</tr>
</tbody>
</table>

2.2.4. Soil nitrogen mineralization potential with ammonium and nitrate determination in microplate format

Soil nitrogen mineralization potential (N-min) was tested according to a modified method of Canali and Benedetti (2006). Samples were assayed in triplicate, with a blank containing no soil sample. Briefly, 200 µl of soil in 1 ml polypropylene (PP) tubes were
incubated for 7 days at 37 °C with 0.25 ml of distilled water and then extracted with 0.25 ml 2M KCl. This consisted of shaking on an orbital shaker at 200 rpm for 30 min at 25 °C in the dark, after which the extracts were centrifuged for 5 min at 1450 g. Subsequently, 200 µl of clear supernatant was transferred to a 96-well polystyrene (PS) microtiter plate and frozen at -20 °C for ammonium and nitrate determination. Triplicate control samples were extracted at the start of incubation with 0.5 ml 1M KCl using the conditions described above. Nitrogen mineralization potential was calculated as the difference in NH₄⁺ (NminNH₄) or NO₃⁻ (NminNO₃) concentration in soil extracts between the control samples and samples incubated for 7 days; results were calculated on the basis of the oven-dry weight of soil, as per Equation 2.4.

\[
N - \min \left( \frac{\text{mg}}{\text{g} \cdot \text{day}} \right) = \frac{7 \text{days}(\text{mg}\text{g}^{-1}) - \text{control}(\text{mg}\text{g}^{-1})}{7(\text{day})}
\]  
(Equation 2.4)

Ammonium determination

In addition to the conventional method of ammonium determination (Chapter 2.2.3), a protocol modified from Diatloff and Rengel (2001) was used for microplate-format ammonium (Amp) determination in soil extracts. Briefly, the following solutions were added to a 96-well PS microtiter plate: 5 µl of 100 mM EDTA, pH = 8.0; 10 µl of 7.8% sodium salicylate with 0.06% sodium nitroprusside in water; 5 of 0.34% sodium dichloroisocyanuric acid in 10% NaOH (after Asaoka et al. 2007) and 30 µl of soil extract. Standard dilutions of (NH₄)₂SO₄ were included in each microplate to construct a calibration curve for estimation of ammonium content. The reaction was left for up to 30 min at room temperature until colour developed. Subsequently, the absorbance of the reaction mixture at 620 nm was determined using a DTX880 plate reader (Beckman Coulter, USA).
Nitrate determination

A modified protocol of Miranda et al. (2001) was used for nitrate determination in soil extracts in microplate format (NO3). Briefly, 50 µl of soil extract in a 96-well PS microtiter plate was combined with 20 µl of 0.4% VCl₃ in 1M HCl and 10 µl of Griess reagent consisting of 0.6% sulphanilamide in 1M HCl and 0.2% N-(1-naphthyl)-ethylenediamine in water mixed in 1:1 ratio (Griess 1879). Standard dilutions of NaNO₃ were included in each microplate to construct a calibration curve for estimation of ammonium content. The plate was incubated for 10 min at 80 °C and the absorbance of the reaction mixture at 535 nm was determined using a DTX880 plate reader (Beckman Coulter, USA).

2.2.5. Catabolic potential

As a measure of metabolic activity of soil organisms, the generation of CO₂ in response to the addition of a C source was assayed using the MicroResp™ approach of Campbell et al. (2003). The substrate set provided in Table 2.2 was modified from Stevenson et al. (2004) according to recommendations (N Banning, pers. comm.). Because of the large number of samples for processing, the MicroResp™ seals were replaced in later experiments by seals prepared “in-house” by punching 96 equally spaced holes in sheets of 3 mm food-grade rubber, which was cut to the size of the incubation plates. As there were routinely more than five sets of incubation-detection plates used in each assay run, softwood spacers were stacked in between plate sets. Clamping of the stacks using woodwork clamps ensured an airtight seal between the plates and seals in each set.

Initially, for the addition of the substrates the MicroResp™ protocol was used, which involved dissolving the substrates in 20 µl of water (Protocol 1). However, for later experiments (Chapters 4 and 5), the protocol was further modified to overcome issues with uneven substrate distribution, varying respiration in soils of different moisture
content and substrate solubility, and long incubation times (Protocol 2). The set of substrates in this latter protocol was reduced to six plus D-glucose and water as controls, totalling eight samples for respiration measurements (Table 2.3).

### Table 2.2. Substrates used in Protocol 1 for the microplate catabolic potential assay.

<table>
<thead>
<tr>
<th>Substrate group</th>
<th>Concentration (mg C ml⁻¹ soil water)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Organic acids</td>
<td></td>
</tr>
<tr>
<td>fumaric acid*</td>
<td></td>
</tr>
<tr>
<td>cinnamic acid</td>
<td></td>
</tr>
<tr>
<td>syringic acid</td>
<td></td>
</tr>
<tr>
<td>urocanic acid</td>
<td></td>
</tr>
<tr>
<td>succinic acid*</td>
<td></td>
</tr>
<tr>
<td>DL-lactic acid</td>
<td></td>
</tr>
<tr>
<td>DL-malic acid*</td>
<td></td>
</tr>
<tr>
<td>formic acid</td>
<td></td>
</tr>
<tr>
<td>glycolic acid*</td>
<td></td>
</tr>
<tr>
<td>L-ascorbic acid</td>
<td></td>
</tr>
<tr>
<td>L-tartaric acid*</td>
<td></td>
</tr>
<tr>
<td>quinic acid</td>
<td></td>
</tr>
<tr>
<td>α-ketobutyric acid</td>
<td></td>
</tr>
<tr>
<td>α-ketoglutaric acid</td>
<td></td>
</tr>
<tr>
<td>Amino acids</td>
<td></td>
</tr>
<tr>
<td>L-asparagine*</td>
<td></td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td></td>
</tr>
<tr>
<td>L-glutamine*</td>
<td></td>
</tr>
<tr>
<td>L-valine*</td>
<td></td>
</tr>
<tr>
<td>L-threonine*</td>
<td></td>
</tr>
<tr>
<td>β-alanine*</td>
<td></td>
</tr>
<tr>
<td>Sugar and alcohols</td>
<td></td>
</tr>
<tr>
<td>mannitol</td>
<td></td>
</tr>
<tr>
<td>L-rhamnose*</td>
<td></td>
</tr>
<tr>
<td>D-galactose*</td>
<td></td>
</tr>
<tr>
<td>Secondary metabolites and vitamins</td>
<td></td>
</tr>
<tr>
<td>coumarin</td>
<td></td>
</tr>
<tr>
<td>quercetin</td>
<td></td>
</tr>
<tr>
<td>(+)-catechin</td>
<td></td>
</tr>
<tr>
<td>imidazole</td>
<td></td>
</tr>
<tr>
<td>thiamine</td>
<td></td>
</tr>
</tbody>
</table>

*Identified root exudates (after Campbell et al. 1997)

### Protocol 1

Briefly, sieved soil was placed in 96-well PS microtiter plates with V-shaped wells of 200 µl capacity, with gentle tapping to ensure equal distribution of the soil sample. Inverting the plate transferred the soil into another 96-well PS microtiter plate (the incubation plate) with 340 µl capacity flat bottom wells containing 20 µl of substrate solutions. Dry weight of 200 µl of soil was determined for each soil sample in three
replicate well after drying at 105 °C for 16 hours. The variability of soil weight between triplicate wells was below 15% (data not shown). Smaller amounts of substrates (3 mg C ml⁻¹ soil water) than indicated in Campbell et al. (2003) and Lalor et al. (2007) (30 mg C ml⁻¹ soil water) were used due to problems with solubility of some of the substrates and also to account for smaller headspace volumes in the modified assay setup.

The detection plate was prepared according to Lalor et al. (2007), with the exception that the detection solution (0.25 M KCl, 4 mM NaHCO₃, 20 µg ml⁻¹ cresol red) contained 0.3% molecular-biology grade agarose (BioRad, Australia) as a gelling agent instead of agar. The solution was heated for a few seconds in a microwave until the agarose dissolved and promptly dispensed into a 96-well PS microtiter plate (100 µl per well). The plate was immediately covered to prevent capture of CO₂ from the air. The incubation plate was sealed with a detection plate using a MicroResp™ or home-made seal immediately after the addition of soil, and was subsequently incubated at 25 °C in dark for up to 6 h. Absorbance of the detection plate at 570 nm was determined using a plate reader immediately before and after the incubation.

Protocol 2

This method used 200 µl of sieved soil dispensed into 96-well microtiter plates, as described above, to which 100 µl of substrate solution was added. Plates were left to equilibrate at room temperature for 1 h as suggested in West and Sparling (1986) and Degens and Harris (1997). Incubation plates were then sealed with detection plates prepared as described above, and incubated for 3 to 6 h in the dark.

Most of the substrates used in this protocol readily dissolved in water, except for cinnamic acid, and were applied at concentrations of 30 mg C ml⁻¹ soil water as suggested in Degens and Harris (1997). Cinnamic acid was used as a saturated solution at less than 3 mg C ml⁻¹ soil water due to its' low solubility in water.
Table 2.3. Substrates used in Protocol 2 for the microplate catabolic potential assay.

<table>
<thead>
<tr>
<th>Substrate group</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic acids</td>
<td>cinnamic acid</td>
</tr>
<tr>
<td></td>
<td>succinic acid</td>
</tr>
<tr>
<td></td>
<td>D-galacturonic acid</td>
</tr>
<tr>
<td></td>
<td>α-ketoglutaric acid</td>
</tr>
<tr>
<td>Sugars and alcohols</td>
<td>D-glucose</td>
</tr>
<tr>
<td>Secondary metabolites and vitamins</td>
<td>imidazole</td>
</tr>
<tr>
<td></td>
<td>thiamine</td>
</tr>
</tbody>
</table>

Data pretreatment

Results were expressed as µg CO₂-C released g soil⁻¹ h⁻¹ (calculated according to calibrations obtained by exposing the detection gel to CO₂ released by strong acid from NaHCO₃ solution). The difference between results obtained from substrate-amended and non-amended samples, expressed as a proportion of the result for non-amended samples was calculated to correct for non-substrate-specific respiration and differences in the magnitude of the response between different soils. This process was repeated for each substrate.

2.2.6. Enzymatic activity assays

Analysis of enzymatic activity of the soil was performed initially in soil slurry amended with buffering solution; however, to balance between assay time, reagents used and sample dispensing efficiency a protocol using whole soil was chosen for later experiments. in order.

Protocol 1 – Whole soil

Soil samples were dispensed into a V-shaped 96-well PS microtiter plate which was inverted over a rack containing 96 x 1 ml PP storage tubes. As a result, each tube contained 200 µl of soil. Tubes were capped and transferred to appropriate assay racks, then 0.5 ml of substrate solutions and buffers were added to provide a suspension of approximately 1:5 (w/v soil:solution). Substrate concentrations are given in Table 2.4.
Assays were typically performed in a set of two replicates with added substrate and one replicate as a no-substrate blank per soil sample. Dry weight of 200 µl of soil was determined for each soil sample in three replicate tubes after drying at 105 °C for 16 hours.

Protocol 2 – Soil slurry

A 1:5 w/v soil slurry was prepared as follows: 30 ml of distilled water was added to 6 g of field-moist soil in a polypropylene container and stirred on a magnetic stirrer for approximately 30 s before dispensing 100 µl of the resulting soil suspension into microtiter plates. An appropriate volume of buffer with or without substrate was added to each well to reach the concentrations given in Table 2.4. The plates were sealed and incubated under the temperature, pH and time conditions indicated in Table 2.4. Assays comprised of three or six test replicates and one or two blanks not containing substrate for each sample. Every assay plate included replicate wells without soil suspension to correct for background substrate decomposition.

After the indicated time, the enzymatic reactions were stopped and products were extracted where appropriate (Table 2.4). Reaction plates or racks were centrifuged briefly to settle the particulates. Some of the assays required detection of the reaction product using additional tests, as indicated in Table 2.4. This was achieved by transferring the appropriate amount of the reaction solution, after centrifugation to remove solids, directly to the detection plate in the same layout as the reaction plate. Series of dilutions of appropriate standards for calibration of the results were added as required. When the additional assays were not performed immediately, as in the cases of urease, cellulase, invertase and xylanase activity, aliquots of reaction mixtures were frozen at -20 °C and assayed within 10 days. In all other cases, absorbance of reaction products in aliquots of cleared supernatants was detected at appropriate wavelengths as indicated in Table 2.4 using a DTX 880 spectrophotometer (Beckman Coulter, USA).

Fluorescein diacetate hydrolysis assay
Total hydrolytic activity of the soil was determined using the fluorescein diacetate (FH) hydrolysis assay as described in Shaw and Burns (2006) with modifications. Briefly, 500 µl of 10 mM phosphate buffer pH = 7.6 was added to 200 µl of field-moist soil in 1 ml PP reaction tubes. Four of 12 subsamples received 20 µg FDA in 10 µl acetone as the substrate. The remaining subsamples received 0, 5, 10 and 20 µg of fluorescein in 10 µl of acetone to provide calibration for the product concentration. Duplicates for each of the four points of the calibration curve were assayed. The subsamples without fluorescein in the calibration curve were treated as no-substrate blanks for the assay. The reaction was done at 25 °C on an orbital shaker set at 250 rpm or on the bench for 30 min and stopped by the addition of 500 µl of acetone to all subsamples. Subsequently, 150 µl of extract was transferred to a microtiter plate and pulse centrifuged to sediment particulates. The absorbance at 485 nm was determined using a DTX 880 spectrophotometer (Beckman Coulter, USA) after transferring 100 µl of the resulting supernatant to an optically clear plate.

**Dehydrogenase activity assay**

The dehydrogenase (DHE) activity assay was done according to Gong (1997) with modifications outlined in Mosher et al. (2003). Briefly, 200 µl of soil was weighed into 1 ml polypropylene tubes and incubated with 100 µl of 0.2% 2, 3, 5-triphenyltetrazolium chloride (TTC) in water at 37 °C without shaking for 24 h. After incubation, 250 µl of acetonitrile was added to the final concentration of approximately 70%, tubes were shaken and particulates were allowed to settle for approximately 10 min at room temperature. Subsequently, 150 µl of extract was transferred to a microtiter plate and centrifuged for 1 min at 1450 g. Aliquots of 100 µl of supernatant were transferred to an optically clear plate for detection of absorbance at 485 nm in the DTX 880 spectrophotometer. The assay consisted of three test replicates and one blank for each soil sample, as well as three test replicates and one blank without soil suspension per sample set to correct for background substrate decomposition.
Procedures for detection of the enzymatic reaction products

Frozen reaction mixtures of assays requiring additional tests for detection of the enzymatic reaction products were defrosted at room temperature for 1 h or at 37 °C for 15 to 30 min.

Cellulase, invertase and xylanase activity

The bicinchoninic acid (BCA) reaction was used for the determination of reducing sugars in the cellulase, invertase and xylanase activity assays. This was performed according to the protocol of Joergensen et al. (1996), but without the addition of an acidifying agent, as no protection from the precipitation of CaSO₄ was needed. Briefly, 10 µl of reaction solution was mixed with 50 µl BCA reagent in a microtiter plate. BCA reagent was prepared as follows: 100 parts of 4% Na₂CO₃, 0.2% DL-aspartic acid in water (pH was adjusted to 11.25) was mixed with 12 parts of 4% BCA dissolved in water and to this mixture 1.8 part of 4% CuSO₄ solution in water was added. The calibration curve for estimating reducing sugars was derived from standard glucose dilutions in triplicate that were included in each plate. The plate was sealed and incubated at 80 °C for a few minutes to allow color development. After incubation, the absorbance at 570 nm was determined using a DTX880 plate reader.

Urease activity

Urease activity was determined using urea as a substrate according to Taylor et al. (2002) with minor modifications, as per Table 2.4. The activity was calculated as the difference in ammonium concentration in the reaction solutions between test and blank replicates. Ammonium concentration was determined according to Diatloff and Rengel (2001) as described above.
Data pre-treatment

Assay results were routinely corrected with two types of blanks: (i) containing no substrate and (ii) containing no soil sample. Results were expressed in μg of product generated, or substrate lost, in case of deaminase activity, per g of oven dry soil per h.

2.3. Statistical analyses

2.3.1. Data limitations

The multivariate data sets resulting from laboratory analyses were highly collinear, often with more variables than observations, and data for many of the variables was not normally distributed, which is often the case for soil and biological studies (Quinn and Keough 2003). This is a problem for classical statistical analysis due to the frequent violation of key assumptions of normality, homoscedasticity and independence of observations (Borcard et al. 2011, Quinn and Keough 2003). Therefore, classical statistical methods of analysis, such as logistic regression, or standard multivariate analysis such as discriminant function and principal component analysis, would be questionable for this type of data set (Quinn and Keough 2003). For the analysis of the data collected throughout the study, a selection of multivariate methods, chosen for the robustness and flexibility with regards to handling non-parametric multivariate data were used.
Table 2.4. Substrates and reaction conditions of microplate assays for testing enzymatic activity in soil.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Unit (g oven dry soil/hr)</th>
<th>Substrate (fc before extraction)</th>
<th>Buffer (fc before extraction)</th>
<th>pH</th>
<th>T (°C)</th>
<th>Time (h)</th>
<th>Extractant (fc after extraction)</th>
<th>Product detection reference</th>
<th>Detection wavelength (nm)</th>
<th>Reference for method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic phosphomonoesterase</td>
<td>µg pNP</td>
<td>1 mM pNP-phosphate</td>
<td>0.5x MUB</td>
<td>6.5</td>
<td>37</td>
<td>3*</td>
<td>50mM CaCl₂, ~0.25M NaOH</td>
<td>-</td>
<td>405</td>
<td>Acosta-Martinez and Tabatabai (2000)</td>
</tr>
<tr>
<td>Alkaline phosphomonoesterase</td>
<td>µg pNP</td>
<td>1 mM pNP-phosphate</td>
<td>0.5x MUB</td>
<td>11</td>
<td>37</td>
<td>3*</td>
<td>50mM CaCl₂, ~0.25M NaOH</td>
<td>-</td>
<td>405</td>
<td>Acosta-Martinez and Tabatabai (2000)</td>
</tr>
<tr>
<td>Arylsulphatase</td>
<td>µg pNP</td>
<td>1 mM pNP-sulphate</td>
<td>50mM NaAcetate</td>
<td>5.2</td>
<td>37</td>
<td>3*</td>
<td>50mM CaCl₂, ~0.25M NaOH</td>
<td>-</td>
<td>405</td>
<td>Acosta-Martinez and Tabatabai (2000)</td>
</tr>
<tr>
<td>Cellulase</td>
<td>µg glucose</td>
<td>0.05% cellulose</td>
<td>50mM NaAcetate</td>
<td>5.2</td>
<td>50</td>
<td>24</td>
<td>-</td>
<td>Joergensen et al. (1996)</td>
<td>570</td>
<td>Schinner and von Mersi (1990)</td>
</tr>
<tr>
<td>Deaminase</td>
<td>µg 1,2-DANB (lost)</td>
<td>0.06% 1,2-DANB</td>
<td>50mM NaAcetate</td>
<td>5.2</td>
<td>37</td>
<td>24**</td>
<td>50% methanol</td>
<td>-</td>
<td>450</td>
<td>Killham and Rashid (1986)</td>
</tr>
<tr>
<td>Invertase</td>
<td>µg glucose</td>
<td>0.05% sucrose</td>
<td>50mM NaAcetate</td>
<td>5.2</td>
<td>50</td>
<td>3*</td>
<td>-</td>
<td>Joergensen et al. (1996)</td>
<td>570</td>
<td>Schinner and von Mersi (1990)</td>
</tr>
<tr>
<td>Phenol oxidase</td>
<td>µg absorbance at 450 nm</td>
<td>2.5 mM L-DOPA</td>
<td>50mM NaAcetate</td>
<td>5.2</td>
<td>25</td>
<td>24</td>
<td>-</td>
<td>-</td>
<td>450</td>
<td>Sayia-Cork et al. (2002)</td>
</tr>
<tr>
<td>Phosphodiesterase</td>
<td>µg pNP</td>
<td>1 mM bis-pNP-phosphate</td>
<td>0.5x MUB</td>
<td>8</td>
<td>37</td>
<td>3*</td>
<td>50mM CaCl₂, ~0.25M NaOH</td>
<td>-</td>
<td>405</td>
<td>Acosta-Martinez and Tabatabai (2000)</td>
</tr>
<tr>
<td>Urease</td>
<td>µg NH₄</td>
<td>100 mM urea</td>
<td>50mM NaAcetate</td>
<td>5.2</td>
<td>25</td>
<td>24+</td>
<td>-</td>
<td>Diatloff and Rengel (2001)</td>
<td>620</td>
<td>Taylor et al. (2002)</td>
</tr>
<tr>
<td>Xylanase</td>
<td>µg glucose</td>
<td>0.05% xylan</td>
<td>50mM NaAcetate</td>
<td>5.2</td>
<td>50</td>
<td>24</td>
<td>-</td>
<td>Joergensen et al. (1996)</td>
<td>570</td>
<td>Schinner and von Mersi (1990)</td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>µg pNP</td>
<td>1 mM pNP-glucopyranoside</td>
<td>0.5x MUB</td>
<td>6</td>
<td>37</td>
<td>3*</td>
<td>50mM CaCl₂, ~0.25M NaOH</td>
<td>-</td>
<td>405</td>
<td>Acosta-Martinez and Tabatabai (2000)</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>absorbance at 485 nm</td>
<td>0.2% TTC</td>
<td>water</td>
<td>-</td>
<td>37</td>
<td>24</td>
<td>70% acetonitrile</td>
<td>-</td>
<td>485</td>
<td>Gong 1997, Mosher et al. (2003)</td>
</tr>
<tr>
<td>FDA hydrolysis</td>
<td>µg fluorescein</td>
<td>FDA</td>
<td>50mM NaAcetate</td>
<td>7.6</td>
<td>37</td>
<td>0.5</td>
<td>50% acetone</td>
<td>-</td>
<td>485</td>
<td>Shaw and Burns (2006)</td>
</tr>
</tbody>
</table>

Abbreviations: fc – final concentration; MUB-Modified Universal Buffer; NaAcetate – sodium acetate; pNP – p-nitrophenol; 1,2-DANB – 1,2-diamino-4-nitrobenzene; L-DOPA – L-3,4-dihydroxyphenylalanine; TTC -2,3,5-triphenyltetrazolium chloride; FDA – fluorescein diacetate. Enzymes involved in nutrient cycling: a – C; b – N; c – P; d – S; * - assay duration using protocol 1 was 1 hr; **- assay duration using protocol 1 was 2 hrs; †- assay duration using protocol 1 was 4 hrs.
2.3.2. Variation partitioning

Variation partitioning was initially developed from partial canonical analysis to assist with the estimation of the spatial component of the variability in ecological data (Borcard et al. 1992). Its use was later extended to modelling the effects of continuous environmental variables or nominal factors on various community composition data sets (Borcard et al. 2011). The procedures and R code described in Borcard et al. (2011) were used throughout the study to estimate the variability in the data sets due to explanatory nominal variables, such as location and treatment, or continuous, such as soil chemical properties. For these latter analyses, the continuous explanatory variables were standardized to the mean of 0 and the variance of 1. For consistency with the distance-based redundancy analysis (db-RDA) models, the response subset was converted to principal coordinates using principal coordinates analysis (Gower 1966) of the dissimilarity matrices calculated with the Bray-Curtis distance measure.

2.3.3. Constrained ordination

Canonical analysis of principal coordinates or distance-based redundancy analysis (db-RDA) is a constrained ordination method which offers considerable advantage over traditional multivariate techniques. It is particularly useful when analysing data sets with variables containing many zeros, skewed distributions and with low number of observations per group (Anderson and Robinson 2003). In the first step, a principal coordinates analysis is performed on a dissimilarity matrix constructed using a chosen dissimilarity measure reduce the number of dimensions in the data. The second step involves a redundancy analysis (Legendre and Anderson 1999) or a linear discriminant function analysis (Anderson and Robinson 2003) with a constraining factor(s) to construct the ordination. This process uses the scores of the principal coordinates as the input data. The significance of the effect of the factor and of the differences between factor levels can be tested using permutation tests, producing results that are analogous to ANOVA (Anderson and Willis 2003). The method has
already been used in the context of soil analyses, for example for soil phospholipid fatty acid profiles (PLFA) measured by Hoyle and Murphy (2006). The Sorensen coefficient, which is the equivalent of the Bray-Curtis distance measure for binary data (Quinn and Keough 2003), was used for redundancy analyses in the study of soil fungal genomes (Kasel et al. 2008). Db-RDA combines the convenience of the graphical representation of the data which facilitates interpretation with the statistical tests of significance of specific hypotheses (Borcard et al. 2011), and as such was deemed most useful for the interpretation of the complex data collected during the current study.

2.3.4. Data preparation and analytical procedures

Whenever a negative result of a laboratory test was observed in the data, the value was treated as 0, and the means of laboratory replicates were calculated. For db-RDA, the variables were typically transformed and ranged (represented as values on a scale from 0 to 1) to reduce the effects of varying scales and non-linearity (Quinn and Keough 2003, Jackson 1993). The Bray-Curtis distance measure was used to calculate the dissimilarity matrices and the partial db-RDA was performed on the square-roots of the matrices, typically with an appropriate factor such as land condition or treatment as the explanatory variable, and the location or site as the conditioning variable. This allowed the analysis of the variability independent of the effects of site or location. The use of the Euclidean distance measure for calculation of the distance matrix from standardized data and a subsequent redundancy analysis (RDA) was also tested. However, the final results, especially the discernment between sample groups in the levels of the constraining variable, were often inferior to those obtained by using the Bray-Curtis measure in db-RDA, so only the latter results were reported. The significance of the effects of the explanatory variables in each db-RDA model was tested using permutation tests in a multivariate ANOVA ($n = 999$ permutations, within the levels of location factor, where appropriate), to provide results analogous to the PerMANOVA (Borcard et al. 2011). The assumption of the homogeneity of the variance-covariance
matrices was tested prior to analyses to assess the validity of the results. This assumption was met in all cases (data not presented). Additionally, the significance of the individual canonical axes was estimated using permutation tests (n = 999) and only the axes significant at the level of $\alpha = 0.05$ were interpreted.

Other statistical methods, such as multiple factor analysis (MFA; Escofier and Pages 1994, Husson et al. 2011) or forward selection of explanatory variables in redundancy analysis (as in Borcard et al. 2011) were used as appropriate and are described in relevant chapters. The analysis and preparation of the graphical output was done using R (version 2.10.1, R Development Core Team 2009) and the Tinn-R program (Faria et al. 2008), R packages “vegan” (Oksanen et al. 2010) and “MASS” (Venables and Ripley 2002) and other packages as indicated in the relevant chapters.
Chapter 3. Soil physical properties have greater impact on soil chemistry than land condition

3.1. Introduction

To effectively manage vegetation to conserve biodiversity, the assessment and monitoring of quantity and quality of native vegetation is important for detecting the impacts of threatening processes and implementing suitable actions (Bleby et al. 2008). Vegetation quality, or “condition”, can be understood as “a state of being or health” (as in Keith and Gorrod 2006). However, since the concept of “vegetation condition” is context-dependent (Bleby et al. 2008), a range of other definitions is available (for review see Bleby et al. 2008, Gibbons and Freudenberger 2006). The use of spatial assessments of vegetation condition as measure of biodiversity is difficult and not always valid, even though they provide often the only biological data that are readily available to estimate the state of an ecosystem and the biodiversity it supports (Bleby et al. 2008, Burgman and Lindenmayer 1998). On the other hand, the results of the on-the-ground vegetation censuses are strongly affected by the observers’ bias, and careful consideration of the sampling design is needed to minimize this shortcoming (Archaux et al. 2007).

Changes in vegetation structure or composition can result in changes in soil properties (Chapter 1). Differences in biochemical soil properties among land conditions have been measured and used successfully to distinguish between areas of different vegetation cover in New Zealand (Macdonald et al. 2009, Stevenson et al. 2004). Changes in enzymatic activity and catabolic potential have been used to monitor the effects of revegetation/restoration in post-mining soils in Cuba (Izquierdo et al. 2005) and Western Australia (Cookson et al. 2008, Lalor et al. 2007). Importantly, significant effects were detected using the microplate format of the catabolic potential assay (Lalor et al. 2007). This result, in addition to the advantages of the microscale methods for soil
testing discussed in Chapter 1, pointed to the usefulness of this methodology for assessing the soil function in the restoration context.

The use of the microplate approach for testing of soil biochemical properties, such as enzymatic activity and catabolic potential, is not yet widely used and has not been optimized for use as a means for assessment of vegetation condition. As a first step in the optimization process, evidence for measurable differences in ecologically relevant soil biochemical properties is required. In this exploratory experiment, the microplate format was used for a range of soil chemical and biochemical properties using soil samples from five land conditions common in the plantation-dominated area of SWWA. The overarching hypothesis was that assays adapted into miniature, high throughput format would detect differences in ecologically relevant soil biochemical properties and allow discernment among these land conditions.

3.2. Materials and methods

3.2.1. Sites description and soil sampling

The study was done using soil samples collected as a part of investigations described by Archibald et al. (2011). Soil samples were collected in late spring (November 2008) from sites Mu, L, C, P, Y, Sq and B (Fig. 2.1), representing five land conditions: (1) intact native woodland which has been altered minimally (IN woodland); (2) remnant patches of native woodland dominated by jarrah (E. marginata) and marri (C. calophylla) with native understorey (UDN) and only moderately altered; (3) remnant patches of native woodland dominated by jarrah and marri with exotic understorey (UDE) and strongly altered; (4) E. globulus plantings (plantation); and (5) managed pasture (pasture). All five land conditions were present in sites C and Y, site Sq and B were of UDN condition, and site P was of UDE condition, while site M lacked UDE and site L lacked UDN.
Soil samples for chemical and biochemical analyses were collected using cores of 5 cm in diameter at sampling depth of 0 to 5 cm, as described by Archibald et al. (2011). Sixteen cores were combined into one composite sample and treated as given in Chapter 2.2. In addition, two cores for bulk density and particle size analysis were collected from areas undisturbed by the sampling for other soil properties.

### 3.2.2. Laboratory methods and techniques

The assays for determining catabolic potential using 30 different substrates were done as described in Chapter 2.2.5. Soil assays were done without adjusting the water content, as the average moisture content was close to 40% of maximum water-holding capacity (maxWHC). Using minimally altered samples was of interest to investigate the microbial response to the moisture as a characteristic of land condition. The nitrogen mineralization potential and enzymatic activity assays were done as described in Chapters 2.2.4 (Protocol 2) and 2.2.6, respectively. Soil bulk density (Bd, g cm\(^{-3}\)) was determined using the standard protocol (Alef and Nannipieri 1995). Particle size analysis was done using a standard hydrometer protocol (Alef and Nannipieri 1995); the percentage results for coarse particles by weight (cpart), sand content (Sand), fine silt content (Silt) and clay content (Clay) are reported. Moisture as a percentage of the maxWHC (MmaxWHC) was calculated based on the measurement of soil water content (M; as percent of wet weight) and maximum water-holding capacity (maxWHC) as per Chapter 2.2.2. Total C: total N ratio (CN) data were also included in the statistical analyses. Soil chemical properties were determined as per Chapter 2.2.3.

### 3.2.3. Statistical analysis

The data from laboratory analyses were treated as described in Chapter 2.3. The data set consisted of 27 samples collected from 7 sites (Chapter 3.2.1) grouped in five land condition groups: IN woodland (n = 4), UDN (n = 6), UDE (n = 9), pasture (n = 4)
and plantation \((n = 4)\). For each group there were 66 variables, which were further grouped into three types, i.e. chemical, biochemical and physical, and five subsets of soil properties (Table 3.1). This division was used consistently in other experiments throughout the study.

The variables were transformed to improve linearity and normality of the data. To balance the design, results were pooled according to location as follows: sites L, Sq and B belonging to location 1; site C to location 2; sites Mu and P to locations 3 and site Y to location 4 (see Figure 2.1). Whenever more samples of the same land condition were present in one location, which was typically the case for the samples collected from the remnants, the data for this land condition were averaged within location. This resulted in a dataset of 20 observations, with 5 land conditions present in each of the 4 locations (Fig.2.1).

The variability in soil chemical and biochemical properties was modeled using soil physical characteristics, land condition and site as explanatory variables. Variation partitioning and distance-based redundancy analysis (db-RDA) were done as described in Chapters 2.3.2 and 2.3.3. Only the most parsimonious partial db-RDA model of the effect of land condition on soil properties is reported in detail.

To investigate the relationships among the different subsets of variables, multiple factor analysis (MFA; Escofier and Pages 1994, Husson et al. 2011) was done on the soil properties measured and additional qualitative factors: land condition and site, using the package “FactoMineR” (Husson et al. 2011). Multiple factor analysis investigates several groups of variables measured on the same sample to determine structures common for groups, while balancing the importance of groups in the global analysis. This method avoids the bias introduced by the group with the highest eigenvalues derived from partial analyses, which would otherwise dominate the global analysis (Escofier and Pages 1994, Becue-Bertaut and Pages 2008).
Table 3.1. Subsets of variables for soil properties investigated in the study. The variables’ names were abbreviated (in parentheses) or given as in Chapter 2 and Chapter 3.2.2.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Biochemical</th>
<th>Physical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium-N (A)</td>
<td>Moisture (M)</td>
<td>Fumaric acid</td>
</tr>
<tr>
<td>Nitrate-N (NitN)</td>
<td>MaxWHC</td>
<td>Cinnamic acid</td>
</tr>
<tr>
<td>Phosphorus (P)</td>
<td>MmaxWHC</td>
<td>Syringic acid</td>
</tr>
<tr>
<td>Potassium (K)</td>
<td></td>
<td>Sand</td>
</tr>
<tr>
<td>Sulphur (S)</td>
<td></td>
<td>Silt</td>
</tr>
<tr>
<td>Organic Carbon (OrgC)</td>
<td>Invertase (IN)</td>
<td>Citric acid</td>
</tr>
<tr>
<td>Total Nitrogen (TotN)</td>
<td>Phenol oxidase (PO)</td>
<td>D-galacturonic acid</td>
</tr>
<tr>
<td>Conductivity (Cond)</td>
<td>Phosphodiesterase (PD)</td>
<td>DL-lactic acid</td>
</tr>
<tr>
<td>pH in CaCl₂ (pH)</td>
<td>N-mineralization potential (Nmin)</td>
<td>DL-malic acid</td>
</tr>
<tr>
<td>C: N ratio (CN)</td>
<td>Urease (UR)</td>
<td>Formic acid</td>
</tr>
<tr>
<td>Ammonium by microplate method (Amp)</td>
<td>Xylanase (XY)</td>
<td>Glycolic acid</td>
</tr>
<tr>
<td></td>
<td>β-glucosidase (BG)</td>
<td>L-ascorbic acid</td>
</tr>
<tr>
<td></td>
<td>Dehydrogenase (DHE)</td>
<td>L-tartaric acid</td>
</tr>
<tr>
<td></td>
<td>FDA hydrolysis (FH)</td>
<td>Quinic acid</td>
</tr>
<tr>
<td></td>
<td>α-ketobutyric acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α-ketoglutaric acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L-asparagine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L-glutamic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L-methionine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L-valine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L-threonine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>β-alanine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mannitol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L-rhamnose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D-galactose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Coumarin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(+)-catechin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Imidazole</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thiamine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank</td>
<td></td>
</tr>
</tbody>
</table>
MFA provides a simplified representation of complex data which can facilitate the interpretation of the results and generation of hypotheses (Escofier and Pages 1994). The number of dimensions retained in the final model was determined using average eigenvalue rule and broken stick model as in Borcard et al. (2011). For clarity of presentation, the significance of the correlations between original variables and the MFA dimensions at the level of $\alpha = 0.01$ or $\alpha = 0.02$ was reported, whichever was available.

3.3. **Results**

3.3.1. **Variation partitioning and db-RDA**

A significant effect of land condition on soil properties was detected even after correcting for the effect of location (Table 3.2). However, when soil physical properties were removed from the response and added to the predictor variables, no independent effect of either land condition or location on the remaining chemical and biochemical soil properties was detected (Table 3.3). Soil physical properties had significant effects on soil chemical and biochemical properties, independent of the effects of land condition and location (Table 3.3).

**Table 3.2.** Variation partitioning in the distance-based redundancy analysis model of the effects of land condition and location on soil physical, chemical and biochemical properties. Bold characters highlight results significant at the level of $\alpha = 0.05$.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Df</th>
<th>Var Model</th>
<th>Var Residual</th>
<th>F</th>
<th>Perm</th>
<th>p value</th>
<th>Adj. R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Land condition</td>
<td>4</td>
<td>0.041</td>
<td>0.114</td>
<td>1.38</td>
<td>999</td>
<td>0.001</td>
<td>0.075</td>
</tr>
<tr>
<td>Location</td>
<td>1</td>
<td>0.010</td>
<td>0.143</td>
<td>1.31</td>
<td>999</td>
<td>0.075</td>
<td>0.016</td>
</tr>
<tr>
<td>Land condition + Location</td>
<td>5</td>
<td>0.052</td>
<td>0.102</td>
<td>1.41</td>
<td>999</td>
<td>0.001</td>
<td>0.101</td>
</tr>
<tr>
<td>Land condition - Location</td>
<td>4</td>
<td>0.041</td>
<td>0.102</td>
<td>1.40</td>
<td>999</td>
<td>0.002</td>
<td>0.085</td>
</tr>
<tr>
<td>Location - Land condition</td>
<td>1</td>
<td>0.010</td>
<td>0.102</td>
<td>1.44</td>
<td>999</td>
<td>0.072</td>
<td>0.026</td>
</tr>
</tbody>
</table>

Abbreviations: Df – degrees of freedom; Var – variance; F – pseudo-F; Perm – number of permutations; Adj. – adjusted.

In the parsimonious model, after correction for the effects of the location, approximately 8% of the variability in soil properties was explained by land condition (Table 3.2). The first two canonical axes (CAPs 1 and 2) were significant (Table 3.4) and
were able to explain up to 70% of this portion the variability, i.e. the variability accounted for by the model.

**Table 3.3.** Variation partitioning in the distance-based redundancy analysis model of the effects of land condition, location and soil physical properties (Structure) on soil chemical and biochemical properties. Bold characters highlight results significant at the level of $\alpha=0.05$.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Df</th>
<th>Var</th>
<th>F</th>
<th>Perm</th>
<th>p value</th>
<th>Adj. R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Land condition</td>
<td>4</td>
<td>15</td>
<td>0.04</td>
<td>0.11</td>
<td>1.42</td>
<td>999</td>
</tr>
<tr>
<td>Location</td>
<td>1</td>
<td>18</td>
<td>0.01</td>
<td>0.14</td>
<td>1.31</td>
<td>999</td>
</tr>
<tr>
<td>Structure</td>
<td>5</td>
<td>14</td>
<td>0.06</td>
<td>0.09</td>
<td>1.84</td>
<td>999</td>
</tr>
<tr>
<td>Land condition + Location</td>
<td>5</td>
<td>14</td>
<td>0.05</td>
<td>0.10</td>
<td>1.46</td>
<td>999</td>
</tr>
<tr>
<td>Land condition + Structure</td>
<td>9</td>
<td>10</td>
<td>0.09</td>
<td>0.06</td>
<td>1.62</td>
<td>999</td>
</tr>
<tr>
<td>Location + Structure</td>
<td>6</td>
<td>13</td>
<td>0.07</td>
<td>0.09</td>
<td>1.71</td>
<td>999</td>
</tr>
<tr>
<td>Land condition +Location+ Structure</td>
<td>10</td>
<td>9</td>
<td>0.10</td>
<td>0.06</td>
<td>1.58</td>
<td>999</td>
</tr>
<tr>
<td>Land condition - (Location+ Structure)</td>
<td>4</td>
<td>9</td>
<td>0.03</td>
<td>0.06</td>
<td>1.21</td>
<td>999</td>
</tr>
<tr>
<td>Location - (Land condition +Structure)</td>
<td>1</td>
<td>9</td>
<td>0.01</td>
<td>0.06</td>
<td>1.08</td>
<td>999</td>
</tr>
<tr>
<td>Structure - (Land condition + Location)</td>
<td>5</td>
<td>9</td>
<td>0.05</td>
<td>0.06</td>
<td>1.46</td>
<td>999</td>
</tr>
<tr>
<td>Land condition - Structure</td>
<td>4</td>
<td>10</td>
<td>0.03</td>
<td>0.06</td>
<td>1.21</td>
<td>999</td>
</tr>
<tr>
<td>Land condition - Location</td>
<td>4</td>
<td>14</td>
<td>0.04</td>
<td>0.10</td>
<td>1.46</td>
<td>999</td>
</tr>
<tr>
<td>Location - Structure</td>
<td>1</td>
<td>13</td>
<td>0.01</td>
<td>0.09</td>
<td>1.03</td>
<td>999</td>
</tr>
<tr>
<td>Location - Land condition</td>
<td>1</td>
<td>14</td>
<td>0.01</td>
<td>0.10</td>
<td>1.44</td>
<td>2999</td>
</tr>
<tr>
<td>Structure - Land condition</td>
<td>5</td>
<td>10</td>
<td>0.05</td>
<td>0.06</td>
<td>1.56</td>
<td>999</td>
</tr>
<tr>
<td>Structure - Location</td>
<td>5</td>
<td>13</td>
<td>0.06</td>
<td>0.09</td>
<td>1.73</td>
<td>999</td>
</tr>
</tbody>
</table>

Abbreviations: Df – degrees of freedom; Var – variance; Mod – Model; Resid – residual; F – pseudo-F; Perm – number of permutations; Adj. – adjusted.

**Table 3.4.** Significance of the canonical axes (CAPs) in the partial distance-based redundancy analysis model of the effects of land condition on soil physical, chemical and biochemical properties, after correction for the effect of location. Bold characters highlight results significant at the level of $\alpha=0.05$.

<table>
<thead>
<tr>
<th>Model</th>
<th>Df</th>
<th>Var</th>
<th>F</th>
<th>Perm</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP1</td>
<td>1</td>
<td>0.315</td>
<td>2.28</td>
<td>999</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>CAP2</td>
<td>1</td>
<td>0.237</td>
<td>1.72</td>
<td>999</td>
<td><strong>0.026</strong></td>
</tr>
<tr>
<td>CAP3</td>
<td>1</td>
<td>0.135</td>
<td>0.98</td>
<td>999</td>
<td>0.373</td>
</tr>
<tr>
<td>CAP4</td>
<td>1</td>
<td>0.100</td>
<td>0.72</td>
<td>999</td>
<td>0.815</td>
</tr>
<tr>
<td>Residual</td>
<td>14</td>
<td>1.935</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Df – degrees of freedom; Var – variance; F – pseudo-F; Perm – number of permutations.

Clear distinction between samples from native and exotic vegetation was observed along CAP1, and between samples from plantations and other land conditions.
along CAP2 (Fig. 3.1 A). Soils from the areas dominated by native vegetation (IN woodland and UDN) were characterised by lower concentrations of nitrate, total N and phosphorus contents, lower activity of urease, β-glucosidase, invertase and xylanase, but a higher C:N ratio. (Fig. 3.1 B). Differences were observed also in soil catabolic potential with soils from areas dominated by native vegetation having higher respiration in response to succinic and α-ketobutyric acids and L-rhamnose, and lower respiration in response to α-ketoglutaric and fumaric acids and D-galactose. The converse was true for the soils from the areas dominated by exotic vegetation (Fig. 3.1 C, D).

Most of the catabolic potential and enzymatic activity measures were lower in plantation soils as compared to other soils tested, while bulk density and phenol oxidase activity was higher (Fig. 3.1 B to D).

### 3.3.2. Multiple Factor Analysis (MFA)

The first seven dimensions of the MFA, which accounted for about 78% of the total variability in the data (Table 3.5), provided a good representation of the whole data set according both to the broken stick model and the average eigenvalue rule (Borcard et al. 2011) (Fig. 3.2 A and B). Dimension 7 was not correlated to the original variables (data not shown), therefore only the first six dimensions are reported in detail.

**Table 3.5.** Proportions of the variation explained by the dimensions in the multiple factor analysis model.

<table>
<thead>
<tr>
<th>Dimension</th>
<th>Proportion of total variation (%)</th>
<th>Cumulative proportion of total variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.60</td>
<td>20.60</td>
</tr>
<tr>
<td>2</td>
<td>14.54</td>
<td>35.14</td>
</tr>
<tr>
<td>3</td>
<td>11.96</td>
<td>47.10</td>
</tr>
<tr>
<td>4</td>
<td>9.14</td>
<td>56.23</td>
</tr>
<tr>
<td>5</td>
<td>8.27</td>
<td>64.50</td>
</tr>
<tr>
<td>6</td>
<td>7.19</td>
<td>71.69</td>
</tr>
<tr>
<td>7</td>
<td>6.52</td>
<td>78.21</td>
</tr>
</tbody>
</table>
Figure 3.1. Differences in physical, chemical and biochemical soil properties between land conditions, detected by partial distance-based redundancy analysis. (A) weighted average samples’ scores in scaling 1, representing Euclidean distances between samples in groups coded by symbols (○ - intact native woodland; ▲- UDN; ● - pasture; ▲ - UDE; ■ - plantation). Dashed lines represent distances from the group centroids; continuous ellipses represent the values of one standard deviation from the group centroids; the percentage of the variability in the db-RDA model explained by the canonical axes is given in parentheses. Relationships between the canonical axes and original variables (weighted average scores in scaling 2) are given separately for moisture and soil chemical properties (in black): moisture (M), maximal water-holding capacity (MaxWHC), moisture as percentage of maximal water-holding capacity (MmaxWHC), ammonium-N (A), nitrate-N (NitN), phosphorus (P), potassium (K), sulphur (S), organic carbon (OrgC), total nitrogen (TotN), conductivity (Cond), pH in CaCl₂ (pH), C: N ratio (CN), ammonium by microplate method (Amp); enzymatic activity (in blue): acidic phosphomonoesterase (AcP), alkaline phosphomonoesterase (AIP), arylsulphatase (AR), cellulase (CE), deaminase (DE), invertase (IN), phenol oxidase (PO), phosphodiesterase (PD), N-mineralization (Nmin), urease (UR), xylanase (XY), β-glucosidase (BG), dehydrogenase (DHE), FDA hydrolysis (FH); physical properties (in red): coarse particles by weight (cpart), sand content (sand), fine silt content (silt), clay content (clay); and catabolic potential substrates (C and D).
Strong correlations between all soil variables subsets were found except for the catabolic potential subset, which was correlated (weakly) only to the chemical variables subset (Table 3.6). Correlation between soil catabolic potential and moisture–related variables was marginally non-significant (Table 3.6).

**Table 3.6.** Correlations among subsets of soil properties in the multiple factor analysis model. Correlation coefficients are given in the bottom left half of the table; the respective p values are given in the top right half of the table.

<table>
<thead>
<tr>
<th>Soil properties</th>
<th>Chemical</th>
<th>Moisture</th>
<th>Enzymatic</th>
<th>Catabolic potential</th>
<th>Physical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical</td>
<td>-</td>
<td>0.003</td>
<td>&lt;0.0001</td>
<td>0.02</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Moisture</td>
<td>0.38</td>
<td>-</td>
<td>0.004</td>
<td>0.08</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>0.55</td>
<td>0.38</td>
<td>-</td>
<td>0.50</td>
<td>0.007</td>
</tr>
<tr>
<td>Catabolic potential</td>
<td>0.38</td>
<td>0.27</td>
<td>0.32</td>
<td>-</td>
<td>0.20</td>
</tr>
<tr>
<td>Physical</td>
<td>0.54</td>
<td>0.51</td>
<td>0.38</td>
<td>0.24</td>
<td>-</td>
</tr>
</tbody>
</table>

The first dimension revealed close relationships between soil physical and chemical properties and enzymatic activity, while the second dimension revealed a relationship to moisture, independent of soil physical and chemical properties (Fig. 3.2C).

Land condition and catabolic potential were strongly correlated to Dimension 3 (Fig. 3.2 D), which separated soil collected from areas under native (IN woodland and UDN) and exotic (pasture and UDE) vegetation, and plantation soils from IN woodland and both UDE and UDN remnant soils (Fig. 3.3 B). The differences were observed mostly for catabolic potential measures, as well as β-glucosidase activity, C: N ratio and soil nitrate content (Fig. 3.3 B and E).

Dimension 4 which grouped enzymatic activity with location, land condition, catabolic potential and soil chemical properties (Fig. 3.2 D), distinguished soil collected from plantation sites from soil collected from pasture sites (Fig. 3.3 B) and revealed that soil pH and respiration in response to addition of the substrates imidazole and L-methionine, were higher, while phenol oxidase activity was lower, in soil from pastures than soil from plantation (Fig. 3.3 B and E).
Figure 3.2. Dimensions of the multiple factor analysis (MFA) assessed using the average eigenvalue rule (A) and broken stick model (B) and the correlations of the soil variables subsets and nominal factors to the first six MFA dimensions (C-E). The percentage of the variability in the MFA model explained by the dimensions is given in parentheses.

Dimension 5 separated both UDE and UDN remnant soils from the other land conditions except plantation (Fig. 3.3 E) and revealed higher N-mineralization and lower respiration in response to addition of the substrates urocanic acid in the remnant soil (Fig. 3.3 C and F). Dimension 6 was not strongly correlated to soil properties and separated soils from UDE remnants, which had lower dehydrogenase activity, from soils from other land conditions except IN woodland and plantation (Fig. 3.3 C and F).
Consistent patterns of location were detected along dimensions 1 to 3, with locations 4 and 1 showing an opposite pattern to locations 2 and 3 (Fig 3.3), and dimension 4, where location 1 was opposed to all others (Fig 3.3 B); however, due to the focus of the current study on the land condition, the details are not reported.

3.4. Discussion

This study presents a direct comparison of chemical and biochemical properties of soils from intact native woodland, native vegetation remnants of varying condition, pasture and *E. globulus* plantation in the SWWA. The results confirmed the presence of differences in soil properties between the five land conditions; however, neither land condition nor location were the main contributors to the variability in the data set. Soil physical properties explained a greater proportion of the variability in the soil chemical and biochemical properties than land condition or location, and, more interestingly, explained most of the variability that could be attributed to these two factors. Thus, land condition and location effects were mediated by the effects of the soil physical properties on soil chemical and biochemical characteristics.

3.4.1. Soil physical properties and the differences among land conditions

In this study, land condition was partly pre-determined by soil physical properties, as, for example, only the areas on native woodland with poorer, gravelly and shallow topsoil were generally uncleared. Biochemical properties and nutrients related to soil microbial activity are linked to soil clay content (Cookson *et al*. 2008b), which in the results of the current study varied along a gradient of sand content, as well as C: N ratio and soil nitrate content. In redundancy analysis, land conditions were ordered along this gradient from intact native woodland, then remnants with native understorey, through plantation to remanets with exotic understorey and pasture. The results suggest that land condition could be tracked by the differences in these properties, which could be of practical use for land managers and restoration practitioners.
Figure 3.3. Differences among land conditions detected by MFA. The MFA scores for the dimensions 1-6 are given in A-C, where the land conditions are coded by symbols: ○ - intact native woodland; ▲ - UDN; ◦ - pasture; ▲ - UDE; □ - plantation. Numbers refer to the four locations sampled during the
study (Chapter 3.2.2). Dashed lines represent distances from the group centroids; continuous ellipses represent the values of one standard deviation from the group centroids; the percentage of the variability in the MFA model explained by the dimensions is given in parentheses. The correlations of the original variables to the dimensions are given with significance at the level of $\alpha = 0.01$ (D, E) and $\alpha = 0.02$ (F). Abbreviations: alkaline phosphomonoesterase (AlP), $\beta$-glucosidase (BG), dehydrogenase (DHE), phenol oxidase (PO), urease (UR), xylanase (XY), deaminase (DE), nitrogen mineralization potential (Nmin), moisture (M), maximal water-holding capacity (MaxWHC), moisture as percentage of maximal water-holding capacity (MmaxWHC), ammonium-N (A), nitrate-N (NitN), phosphorus (P), organic carbon (OrgC), total nitrogen (TotN), conductivity (Cond), pH in $\text{CaCl}_2$ (pH), C: N ratio (CN), bulk density (Bd), sand content (sand), fine silt content (silt), clay content (clay).
Some physical properties were likely to have been influential in determining land use, for example farmers would have made judgements that may have considered soil properties (directly or indirectly) when deciding areas to be cleared for pasture, and which would be left intact as remnants. Other properties were influenced by processes involved in land use change, for example bulk density, which increases as a result of grazing by sheep and cattle (Yates et al. 2000b). In the current study, plantation soils had higher bulk density than pasture soils, even though grazing pressure had been removed. Moreover, the activity of enzymes was lower in the plantation soils, which was probably due to lower moisture content; however, negative effects of increased soil bulk density on enzymatic activity have also been observed for at least one enzyme in other studies: alkaline phosphomonoesterase (Kissling et al. 2006). The difficulty in inferring the causality in cases such as this one stems from the design of the current study, which, due to the practical considerations, was not a “before-and-after” study in relation to land use change. This inescapable shortcoming was unfortunate, considering the importance of understanding what changes occur in which soil properties during land use change and why, so that the restoration efforts could be specifically targeted to reversing them.

The MFA, a more general and descriptive method for detecting associations between variables, provided similar results to the hypotheses-testing db-RDA. Moreover, it allowed an analysis “constrained” by the land condition and location, which forced detection of structures characteristic to the influences of these two factors. Such treatment detected specific combinations of variables, clearly separating the land conditions and suggested certain assays for discerning between the land conditions. MFA revealed the relative importance of catabolic potential in detecting differences among land use types compared to other methods, which agrees with other research (Cookson et al. 2008a, Lalor et al. 2007).

In this study, the factor of land condition encompassed also the variability in vegetation type, i.e. exotic versus native. The impact of vegetation type on soil
biochemical properties is well documented (for example Singh et al. 2009, Allison et al. 2006, Zak et al. 2003). However, contrary to expectations, the results of the current study suggested that the effects of the vegetation type on soil biochemistry were masked by those of soil physical characteristics. This could be due to relatively large differences in soil bulk density and texture between the extreme land use types, for example between intact native woodland and pasture. Consequently, the effects of vegetation type on soil biochemistry could possibly become clearer if experiments were performed on soils with more homogenous physical properties. Also, the effects of plants on soil microbiota are observed in rhizosphere soils rather than in bulk soils, and the difficulty in separating these two, inherent in the field studies, may have been another factor confounding the results.

**3.4.2. Differences in soil properties among land use types**

The land condition factor explained only a small portion of the variability in the collected data, suggesting that the effects of the land condition, especially when corrected for the soil physical properties, are relatively small. The MFA analysis confirmed this by detecting the structures characteristic to the land use types in the later dimensions (3 through to 6) rather than in the first two. The placement of the remnants soils between the intact native woodland and pasture with plantation soils agreed with the assumption that the differences in vegetation would correspond to differences in biochemical soil properties, suggesting that soil analyses could provide a means to assess vegetation condition. Plantation soils differed from the soils from other land conditions, suggesting that planting exotic trees induced characteristic changes in soil biochemical properties. Higher compaction, lower moisture and pH were likely reasons for reduced respiration and enzymatic activity observed in the plantation soils. Increased phenol oxidase activity could be expected as a result of mostly lignin-based litter being essentially the dominant substrate available in the plantations (Briones and Ineson 1996). plantation soils were also markedly lower in nitrate N, K, ammonium and S than
other soils, probably due to nutrient uptake by the trees. Planting trees to reduce nutrient enrichment in post-pasture soils has been suggested as a restoration method by Cole et al. (2004). Plantation soils were similar to pasture according to MFA and no clear shift in chemical and biochemical soil properties towards the IN woodland were detected. However, based on the db-RDA results, in at least some soil properties, for example in the activity of carbon cycling enzymes and N contents, such a shift occurred, which would cautiously support the hypothesis of Cole et al. (2004).

In the current study, a small number of microplate tests for soil biochemical properties could clearly discern between the UDE and UDN remnants. The MFA and db-RDA analyses confirmed the differences in soil biochemistry between these two land use types and revealed that C:N ratio, nitrate contents, β-glucosidase activity and respiration in response to succinic and α-ketobutyric acid were strongly related to differences between soils under native versus exotic, understorey. Close et al. (2008) reported lower C: N ratio in heavily, compared to lightly, grazed remnants in Tasmania and Zornoza et al. (2009) reported higher C: N ratio in soils from native forest as compared to agricultural land in Spain. The differences in the latter study were attributed to the characteristics of the litter, with that from native vegetation being much more recalcitrant than that of agricultural plants. Increased nitrate levels in soil were associated with invasion of exotic plant species in urban remnants in Melbourne, Australia (Bidwell et al. 2006) and also with heavy disturbance by grazing in Tasmania (Close et al. 2008). In addition, the activity of soil β-glucosidase has been proposed as an efficient indicator of C-cycling efficiency in an agricultural system (Lagomarsino et al. 2009). The results of the current study follow these patterns observed in various other systems.

The differences in soil properties between remnants with exotic understorey and pasture, and between intact native woodland and remnants with native understorey were not clear; however, they were apparent as trends in db-RDA. Based on MFA, the differences were mostly in respiration in response to urocanic acid, which was a
substrate that Nsabimana et al. (2004) found to be characteristically lower in exotic forests than in other land uses investigated in their study. In the current study, both types of remnants had lower rates of respiration in response to this substrate than intact native woodland and pasture, but not plantation, which suggested that the characteristic combinations of soil properties common for IN woodland and pasture still exist.

Moreover, in the current study, patterns in the data common for remnants with exotic understorey and intact native woodland were detected. Differences in soil nutrient pools as a result of a change in vegetation can be detected for up to 120 years after the land use change has occurred (Compton and Boone 2000). Thus, soil chemical properties are invaluable in conducting historical studies of the past vegetation covers (Koerner et al. 1999) and monitoring land use changes. In the current study, the relatively shorter-term changes can also be detected using soil biochemical properties, and that this may be useful as a tool to measure soil improvement or degradation over time, even if only on a time-scale of decades.

The interpretation of Dimension 5 of MFA revealed a small number of soil properties characteristic of the differences between intact native woodland and remnants. For example, remnant soils had higher N-mineralization potential than the IN woodland and pasture soils. The results could also suggest that fragmenting the landscape by creating a remnant from a larger patch of woodland impacts on soil properties even without marked changes in understorey vegetation. This could be due to loss of specific plant species with strong impact on soil biochemical properties; however, to the best of the author's knowledge, detailed studies of such effects in WA are not available to date. Morris and Boerner (1999a) detected higher levels of N-mineralization potential in cleared areas of the temperate forest. O'Connell et al. (2003) reported a decrease in soil N-mineralization and N supply in the pastureland converted to the E. globulus plantations in WA, due to changes in organic matter quality and soil moisture, which was not apparent in the current study. The results of the present study suggest
that similar changes may be occurring with the transition from IN woodland to UDN, potentially as a result of a decrease in tree density or changes in vegetation composition after fragmentation.

3.5. **Conclusions**

Soil chemical and biochemical properties, tested in microplate format, alongside the soil physical properties, allowed discernment of all five land conditions, all of which represented different degrees of departure from an intact native woodland state. Physical soil characteristics had major influence on chemical and biochemical properties, which contributed to difficulties in disentangling multiple interrelated factors after a disturbance event such as land use change. Manipulative experiments leading to collection of “before-and-after” data would elucidate this process. Several such experiments will be presented in the following chapters. The results also revealed a range of assays helpful in discerning between soils from different land use types, including between remnants of varying conditions. This provides a basis for more in-depth investigations, which could lead to a better understanding of the processes involved in shifting the characteristic of the soils in remnants with exotic understorey towards those observed in remnants with native understorey, and thus to better restoration practices. The importance of such investigations lies in their potential to elucidate the biological processes in soil to inform land management and restoration practices while providing efficient laboratory methods for monitoring outcomes.
Chapter 4. Effects of short term nutrient enrichment, weed invasion and physical disturbance on vegetation and biochemical soil properties in native woodland remnants

4.1. Introduction

Clearing and subsequent conversion of native vegetation to agricultural land has had a strong and negative impact on Australian landscapes, causing landscape change and biodiversity loss (Lindenmayer and Fisher 2006, Yates and Hobbs 1997). Agricultural clearing has caused fragmentation of native woodlands and has created patches of vegetation that often are all that remain to preserve the structure and diversity of the assemblages of native plant and animal species (Yates and Hobbs 1997). Such remnants, if undisturbed, are local repositories of indigenous biodiversity; however, both in WA and worldwide, they are subject to gradual decline, manifesting primarily as a loss of plant species (for example Archibald et al. 2011, Close et al. 2008, Oliveira et al. 2008).

Changes in plant communities, including the loss of species, can alter soil microbial communities, especially in soils that are low in organic matter (Zak et al. 2003), such as those commonly found in WA (Qiu et al. 2008). Nutrient enrichment is a degradation factor in nutrient-poor soils, favouring the persistence of annual exotic plant species over perennial natives (Davidson et al. 2008, Spooner et al. 2002). With neighbouring monocultures of exotic plants and a reliance on artificial fertilizers, agricultural systems can contribute to major changes in the cycling of nutrients in adjacent remnant woodlands, for example, thorough changes in litter quality and levels of N input from exotic plantings (Allison et al. 2006). Changes in soil nutrient pools can also occur as a result of vegetation clearing alone (Wu et al. 2011, Morris and Boemer 1999a).
The effects of different land management practices can be mediated by soil microbes and, as a consequence, can be detected by measuring changes in soil microbial activity (for example Lagomarsino et al. 2009, Kandeler et al. 1999). Understanding changes in soil function resulting from degradation processes would enable more efficient monitoring and interventions, including restoration.

The experiment described in Chapter 3 demonstrated that soil biochemical properties varied depending on land condition. Clear differences between pasture and native forest were measured. More interestingly, marked differences between the biochemical characteristics of the soils from the remnants with native (UDN) and exotic (UDN) understorey were also detected, suggesting that biochemical soil characteristics could be used for investigating the impact of land use change. However, a bias caused by the relationships between soil physical characteristics and chemical and biochemical soil properties limited the scope of inference from this study and called for another experiment, where variability due to soil physical properties would be controlled for.

Detailed studies focusing on changes in soil biochemical properties due to the replacement of native plant communities with exotic species and artificial fertilization in the SWWA are, to our knowledge, not available. Addressing this knowledge gap is especially important for areas where the need for restoration of native plant communities exists, such as in remnants in *E. globulus* plantations. The aim of the current experiment was to obtain information about biochemical properties of the soil as indicators of degradation processes. Suitable indicators could then be used in monitoring of the land condition and for assessment of the efficiency of rehabilitation treatments. The hypothesis was that that the changes in vegetation cover and soil biochemical and chemical properties would occur with the following: a) removal of understorey vegetation and leaf litter and raking of the soil, representing artificial clearing; b) fertilization with N, P and K, and c) the establishment of exotic grass species, such as annual and perennial ryegrass, and that the changes in soil biochemical and chemical properties would be more pronounced with fertilization than with the other treatments. This was tested by
analysing soils for a range of chemical properties using conventional approaches and a series of biochemical soil properties using microplate-format assays. The changes in native and exotic vegetation cover, resulting from the experimental treatments, were also investigated to determine which of the degradation factors, simulated by the treatments, had the greatest impact.

4.2. Experimental design and procedures

4.2.1. Study sites

Three remnants of native jarrah forest, each within a different *E. globulus* plantation matrix and within 20 km from one another (sites Mi, R and RG, Figure 2.1, Chapter 2) and with overstorey canopy cover of approximately 20 to 50% were selected. The shrubby understorey of the three remnants was dominated by *Agonis theiformis* and the ground cover was mostly leaf litter and native herbs, with less than 1% coarse woody debris and rocks. The understorey of site R was least dense (<10% of ground cover by shrubs taller than 2 m) and included *Banksia grandis*, while that of site RG included *Allocasuarina* spp. and was the densest of the three sites, with over 60% of foliar cover by shrubs taller than 2 m. Soil characteristics and nutrient levels for the study sites are given in Table 4.1.

4.2.2. Experimental treatments

Within each of three remnants, fifteen circular treatment plots of 2 m diameter, with similar vegetation cover and not encompassing trees (i.e. stems thicker than 1 cm), were randomly selected. No exotic plant species were observed in the vicinity of the experimental plots within the sites. The treatments aimed at mimicking the factors known to contribute to remnant degradation in the area from grazing by livestock, namely: clearing of native vegetation, increased soil nutrient content and invasion of exotic plant species (Archibald *et al.* 2011). Plots were randomly assigned to one of the following five
treatments: 1. non-treated control; 2. cleared; 3. cleared and fertilized; 4. cleared and seeded with ryegrass; 5. cleared, fertilized and seeded with ryegrass (Table 4.2). Each treatment was replicated three times in each site (remnant).

The treatments were applied in April 2009, at the start of winter. Clearing involved cutting and removal of stems, removal of leaf litter to expose mineral soil and raking of the soil surface. Garden fertilizer “NPK Blue” (Cresco, composition: 13.6% N (ammonium 12.6%); 1.9% P; 8% K, 14.2% S and micronutrients: Ca 2.6%, Zn and Cu 0.08%; Mg, Fe and Mn - 0.3%) was used at 500 kg ha⁻¹ for the fertilizer treatment, as recommended by the manufacturer for topical application in gardening. For the ryegrass treatments, a 1:1 v/v mixture of annual and perennial ryegrass (*Lolium rigidum* and *Lolium perenne*; respectively) at 0.5 kg seed m² was used. The seed was dispersed evenly across the plot by hand into raked soil.

4.2.3. Data collection

Ground cover surveys and soil collection were done before treatment application in April 2009 (before the beginning of wet season), and 3 and 6 months after, i.e. in July and October 2009, in the middle and at the end of the wet season, respectively. At each assessment, the number of native plant species (NS), percentage of ground covered by green plants: native (NC) and exotic (EC) species, and the average height of the exotic (ryegrass) cover (EH) within plots were estimated twice: once by each of the two observers. Additionally, the maximum height of ryegrass (MEH) and native vegetation regrowing from epicormic shoots and newly emerging germinants (NH) was recorded at 6 months after treatment application.
Table 4.1. Mean values ±SD of the selected chemical properties of the soil in the three study sites.

<table>
<thead>
<tr>
<th>Soil properties (abbreviation)</th>
<th>Unit</th>
<th>Reference</th>
<th>Site</th>
<th>Mi</th>
<th>R</th>
<th>RG</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH in CaCl₂ (pH)(^a)</td>
<td></td>
<td>Rayment and Higginson (1992)</td>
<td>3.80 ± 0.16</td>
<td>4.30 ± 0.17</td>
<td>4.60 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>Organic Carbon (OrgC)(^a)</td>
<td>%</td>
<td>Walkley and Black (1934)</td>
<td>5.50 ± 0.80</td>
<td>4.10 ± 0.50</td>
<td>4.32 ± 1.00</td>
<td></td>
</tr>
<tr>
<td>Total Nitrogen (TotN)(^a)</td>
<td>%</td>
<td>Rayment and Lyons (2011)</td>
<td>0.26 ± 0.04</td>
<td>0.22 ± 0.06</td>
<td>0.17 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Moisture (M)(^b)</td>
<td>%</td>
<td>Buurman et al. (1996)</td>
<td>8.93 ± 1.49</td>
<td>8.80 ± 2.34</td>
<td>12.5 ± 2.00</td>
<td></td>
</tr>
<tr>
<td>Exchangeable potassium -Colwell (K)(^a, b)</td>
<td>mg kg(^-1)</td>
<td>Rayment and Higginson (1992)</td>
<td>99.8 ± 23.54</td>
<td>109.7 ± 30.34</td>
<td>80.7 ± 37.54</td>
<td></td>
</tr>
<tr>
<td>Exchangeable phosphorus -Colwell (P)(^a, b)</td>
<td>mg kg(^-1)</td>
<td>Rayment and Higginson (1992)</td>
<td>4.92 ± 1.03</td>
<td>3.50 ± 0.99</td>
<td>4.12 ± 0.64</td>
<td></td>
</tr>
<tr>
<td>Conductivity (Cond)(^b)</td>
<td>dS m(^-1)</td>
<td>Rayment and Higginson (1992)</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.02</td>
<td>0.08 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Sulphur (S)(^a)</td>
<td>mg kg(^-1)</td>
<td>Blair et al. (1991)</td>
<td>4.94 ± 2.10</td>
<td>5.00 ± 1.24</td>
<td>5.34 ± 1.17</td>
<td></td>
</tr>
<tr>
<td>Ammonium- N (A)(^a, b)</td>
<td>mg kg(^-1)</td>
<td>Searle (1984)</td>
<td>5.62 ± 1.72</td>
<td>5.70 ± 2.81</td>
<td>5.13 ± 1.67</td>
<td></td>
</tr>
<tr>
<td>Nitrate - N (NitN)(^a, b)</td>
<td>mg kg(^-1)</td>
<td>Searle (1984)</td>
<td>&lt;1 na &lt;1 na</td>
<td>&lt;1 na</td>
<td>&lt;1 na</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) - below detection limit; \(^b\) - analyses performed by Cumming Smith British Petroleum Plant and Soil Laboratory (CSBP) in Bibra Lake, WA; \(^b\) - analyses performed for soils collected at three months after treatment application; na – not available.

Table 4.2. Experimental treatments in the study investigating the degradation factors in three remnants of native woodland within E. globulus plantations in the SWWA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Clearing (with litter removal)</th>
<th>Fertilizer (500kg ha(^-1))</th>
<th>Ryegrass (0.5 kg m(^2))</th>
<th>Replicates per site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-treated control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Cleared control</td>
<td>✓</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Cleared +Fertilizer</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Cleared +Ryegrass</td>
<td>✓</td>
<td>-</td>
<td>✓</td>
<td>3</td>
</tr>
<tr>
<td>Cleared +Fertilizer + Ryegrass</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>3</td>
</tr>
</tbody>
</table>
Groundcover assessments were made prior to soil collection. Soil sampling involved collecting 12 soil cores (5 cm in diameter and 5 cm depth) from each plot using a steel tube. A sampling grid ensured a unique location was sampled each time, thereby avoiding the sampling of soils disturbed by the previous collection. Samples were transported to the laboratory in sealed plastic bags at ambient temperature and analysed for the activity of soil alkaline phosphomonoesterase (AP), urease (UR), arylsulphatase (AR), deaminase (DE) and β-glucosidase (BG) as described in Chapter 2.2.6 (Protocol 1). Soil respiration induced by water (w) and substrates: D-glucose (g), D-galacturonic acid (ga), succinic acid (sa), α-ketoglutaric acid (k), imidazole (i) and thiamine (t), at concentrations of 30 mg carbon ml⁻¹, and cinnamic acid (c) at less than 3 mg carbon ml⁻¹, was assayed as described in Chapter 2.2.5 (Protocol 2). Soil chemical properties were determined as described in Chapter 2.2.3. Soil nitrogen mineralization potential assay and ammonium (Amp) and nitrate (NO₃) determination in microplate format was done as described in Chapter 2.2.4, except for the sampling before treatment application, where nitrate determination was omitted due to laboratory error.

4.2.4. Statistical analyses

The effects of experimental treatment on NC, EC and NS were investigated using univariate two-way fixed factors ANOVA with site as a second factor. Due to the focus of the current study, only the results for the treatment factor are reported. The analysis was done separately for data sets from 3 and 6 months after treatment application, with n = 9 replicates per treatment group. Tukey's Honestly Significant Difference test was applied to correct for multiple comparisons; significance was assumed at p < 0.05. To investigate the effects of site on vegetation growth after clearing, a separate analysis of site effect on native and exotic plant cover was conducted for each of the three data sets using ANOVA with Tukey's Honestly Significant Difference test for multiple comparisons, with experimental treatment as the second fixed factor, where applicable. In this case, only
the data from the treated plots was used, resulting in sample sizes of 12 and 6 per treatment level for native and exotic plant cover, respectively.

The effects of the experimental treatments on soil and vegetation related variables were tested using variation partitioning and partial distance-based RDA as described in Chapter 2.3. The analyses were done using the data collected before (0 months) and after 3 and 6 months after treatment application. All partial models were conditioned for the effects of site. Since the results of the nitrate N content of soil obtained from the external laboratory were below detection limit (<1 g kg soil\(^{-1}\)) in all samples tested, they were not included in statistical analyses. All variables were pre-treated as given in Chapter 2.3. The effect of treatment as a single factor with five levels (non-treated control; cleared control; cleared and fertilized; cleared and seeded with ryegrass; cleared, fertilized and seeded with ryegrass) was tested. A simultaneous analysis of the effects of the three degrading factors in a fully factorial design was not possible due to the unbalanced design of the study. To overcome this issue, the 3 and 6 months data sets were split into two subsets for analysis as follows: the effect of clearing was tested in a one-way design, using samples obtained from control plots, i.e. non-treated and cleared, with n = 9 replicates each, i.e. three plots in each of the three sites (Table 4.2); the effect of fertilizer and ryegrass establishment was tested using a two-way factorial design using samples obtained from all cleared plots, i.e. all plots except non-treated controls, with n = 9 replicates each. To detect the effects of fertilizer on soil biochemical properties, aside from the expected increase in soil N, P, K and S content, partial models with and without conditioning for these four variables were tested. For the analysis of the pre-treatment data set, dummy treatments were randomly assigned to the plots to ensure that the differences in later data sets were not due to the variability already present in soil biochemical properties before the treatments were applied. Additionally, the variability between samples and relationships between variables in this data set was investigated using principal components analysis.
4.3. **Results**

4.3.1. **Differences in plant cover**

Native plant cover did not reach pre-clearing levels of vegetation cover after treatment application; however, it was significantly higher in treated plots at 6 than at 3 months, with bigger differences in ryegrass-treated plots, both with and without fertilizer application (Fig. 4.1 A).

Ryegrass treatment resulted in an increase of up to 80% of exotic plant cover in cleared plots at 3 months, but only 40% at 6 months after treatment application; fertilization did not provide additional increases (Fig. 4.1 B). The average height of ryegrass was about 5 cm in unfertilized plots and up to three times that in the fertilized plots at both 3 and 6 months after treatment application (data not shown), which was a probable cause of the greener appearance of the cover at 3 months after treatment application (Fig. 4.2). The number of native plant species re-sprouting after clearing, was significantly lower in plots with fertilization and ryegrass treatment (Fig. 4.1 C). However, 6 months after treatment application, the number of native plant species counted in the treated plots was similar to that in the control plots (Fig. 4.1 C).

Significant differences in native plant cover among sites were observed at the beginning of the trial, but not at 3 months after treatment application (Table 4.3). At 6 months, native vegetation cover in cleared plots differed significantly between sites; however, in a different pattern (Table 4.3). Native plant cover did not re-sprout to the pre-clearing levels during the study (Table 4.3). Significant and consistent differences in exotic plant cover were found at 3 and 6 months after treatment application, with values higher at 3 than at 6 months (Table 4.3).
Figure 4.1. Effect of the experimental treatments on native plant cover (A), exotic plant cover (B) and number of native plant species (C) at 3 and 6 months after treatment application. Mean values from 9 plots per treatment with standard error of the mean (error bars) are given; * - p <0.05, ** - p <0.01. Different letters denote significant differences from non-treated control (p <0.05) at 3 months (lower case) and 6 months (upper case) after treatment application.
Table 4.3. Native (NC) and exotic (EC) ground cover (%) in the study sites (Mi, RG and R) before and at 3 and 6 months after treatment application. Mean values ±SD for the treated plots averaged across treatments are given, with statistical comparisons when possible. Within columns, values followed by the same letter are not significantly different at the level of α = 0.05.

<table>
<thead>
<tr>
<th>Ground cover measure</th>
<th>Site</th>
<th>Months after treatment application</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>NC (n = 12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mi</td>
<td>37.9</td>
<td>± 12.7</td>
</tr>
<tr>
<td>RG</td>
<td>75.4</td>
<td>± 9.9</td>
</tr>
<tr>
<td>R</td>
<td>23.3</td>
<td>± 12.7</td>
</tr>
<tr>
<td>Statistical comparison</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F*</td>
<td>62</td>
<td>2.72</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.001</td>
<td>0.09</td>
</tr>
<tr>
<td>EC (n = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mi</td>
<td>nd</td>
<td>na</td>
</tr>
<tr>
<td>RG</td>
<td>nd</td>
<td>na</td>
</tr>
<tr>
<td>R</td>
<td>nd</td>
<td>na</td>
</tr>
<tr>
<td>Statistical comparison</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F_{2,12}</td>
<td>na</td>
<td>34.6</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Abbreviations: nd – not detected; na – not available. * - F_{2,33} for 0 months after treatment application, one way ANOVA; F_{2,24} for 3 and 6 months after treatment application, two-way ANOVA with fixed factors.

4.3.2. The effects of experimental treatments on soil properties and vegetation – one factor

No differences in soil properties among experimental plots that were assigned the treatments were found before treatment application when tested either in whole or in split data sets (data not shown). Principal components analysis revealed grouping along the first principal component (PC1) according to site, with soils from site Mi having higher totN, OrgC, P and K concentrations, as well as ammonium (measured by conventional method). Respiration in response to glucose and imidazole, activity of deaminase, β-glucosidase and urease was also higher in Mi than in soils from other sites (Fig. 4.3). Soil from RG had higher pH, greater activity of alkaline phosphomonoesterase and arylsulphatase and higher ammonium concentration (measured in microplate format) than soils from other sites. The two first principal components explained only 40.6% of the total variability. No clear groupings according to treatment were detected by PCA at the end of the experiment (data not shown).
Figure 4.2. Representative plots in site RG at three months after application of treatments mimicking degradation factors: (A) Non-treated control; (B) Cleared, identical to Cleared + Fertilizer treatment; (C) Cleared + Ryegrass; (D) Cleared + Fertilizer + Ryegrass.
Experimental treatments had a significant effect on soil properties and vegetation, and explained 23 and 14% of total variability in the data sets at 3 and 6 months after treatment application, respectively (Table 4.4).

**Table 4.4.** Effects of experimental treatments on soil properties and vegetation at 3 and 6 months after treatment detected by variation partitioning.

<table>
<thead>
<tr>
<th>Months after treatment application</th>
<th>Factor</th>
<th>Df Model</th>
<th>Residual</th>
<th>Adjusted R²</th>
<th>Pseudo-F</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Treatment</td>
<td>4</td>
<td>40</td>
<td>0.21</td>
<td>3.91</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Site</td>
<td>2</td>
<td>42</td>
<td>0.11</td>
<td>3.60</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Treatment + Site</td>
<td>6</td>
<td>38</td>
<td>0.34</td>
<td>4.73</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Treatment - Site</td>
<td>4</td>
<td>38</td>
<td>0.23</td>
<td>4.67</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Site - Treatment</td>
<td>2</td>
<td>38</td>
<td>0.13</td>
<td>4.85</td>
<td>0.001</td>
</tr>
<tr>
<td>6</td>
<td>Treatment</td>
<td>4</td>
<td>40</td>
<td>0.12</td>
<td>2.52</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Site</td>
<td>2</td>
<td>42</td>
<td>0.11</td>
<td>3.70</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Treatment + Site</td>
<td>6</td>
<td>38</td>
<td>0.25</td>
<td>3.43</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Treatment - Site</td>
<td>4</td>
<td>38</td>
<td>0.14</td>
<td>2.95</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Site - Treatment</td>
<td>2</td>
<td>38</td>
<td>0.13</td>
<td>4.38</td>
<td>0.001</td>
</tr>
</tbody>
</table>

The first three canonical axes were significant at both of these sampling times (Table 4.5) and provided separation between the five experimental treatments, which was clearer at 3 than at 6 months after treatment application (Fig. 4.4 A, C and 4.5 A, C). Variables related to the vegetation and to soil nutrients were most highly correlated to the first and second canonical axis (CAP1 and CAP2), respectively (Fig. 4.4 B, D and 4.5 B, D). Cover of exotic plants was negatively related to higher levels of ammonium-N, P and K, which were present in soil from fertilized, compared to non-fertilized plots (Fig. 4.4 B, D and 4.5 B, D). The third canonical axis (CAP3) was related to the effect of clearing and separated the non-treated and the cleared control plots (Fig. 4.4 C, D and 4.5 C, D).
Figure 4.3. First two principal components of the analysis of soil properties before treatment application. Symbols code for sites: - Mi; - R; - RG. Lines represent components’ loadings. Abbreviations: Nmin- nitrogen mineralization potential, Amp- ammonium by microplate method, M- moisture, A -ammonium-N, P - phosphorus, K- potassium, S- sulphur, OrgC- organic carbon, TotN- total nitrogen, Cond- conductivity, pH- pH in CaCl₂; enzymatic activity of: AP- alkaline phosphomonoesterase, UR- urease, AR- arylsulphatase, DE- deaminase, BG-β-glucosidase; respiration induced by: w- water, g- D-glucose, ga- D-galacturonic acid, sa- succinic acid, k- α-ketoglutaric acid, i- imidazole, t- thiamine, c- cinnamic acid.

4.3.3. The effects of experimental treatment on soil properties and vegetation – three factors

Variation partitioning analysis of the data sets split by treatment revealed no effects of three experimental treatments on soil properties except for elevated levels of soil nutrients in the fertilizer treatment at both 3 and 6 months after treatment application.
(data not presented). When the variables NO₃, Amp and A (ammonium content measured using microplate and conventional method, respectively), P, K and S (constituents of the fertilizer added) were excluded from the response data sets, application of fertilizer had a significant effect ($p = 0.005$) on the remaining soil properties at 6 months after treatment application (Table 4.6), with higher β-glucosidase activity and catabolic potential (excluding control), and lower urease and arylsulphatase activities in the treatments involving fertilizer application (Fig. 4.6).

Table 4.5. Significance of the canonical axes (CAPs) in the distance-based redundancy analysis of the effects of experimental treatments on soil properties and vegetation at 3 and 6 months after treatment application. Bold characters highlight values significant at the level of $\alpha = 0.05$.

<table>
<thead>
<tr>
<th>CAP</th>
<th>Df</th>
<th>Months after treatment application</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>6</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Variance</td>
<td>Pseudo-F</td>
<td>p value</td>
<td>Variance</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0.019</td>
<td>11.08</td>
<td><strong>0.001</strong></td>
<td></td>
<td>0.018</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.009</td>
<td>5.27</td>
<td><strong>0.001</strong></td>
<td></td>
<td>0.006</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0.003</td>
<td>1.56</td>
<td><strong>0.015</strong></td>
<td></td>
<td>0.004</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0.001</td>
<td>0.76</td>
<td>0.912</td>
<td>0.002</td>
<td>0.90</td>
</tr>
<tr>
<td>Residual</td>
<td>38</td>
<td>0.067</td>
<td></td>
<td></td>
<td></td>
<td>0.970</td>
</tr>
</tbody>
</table>

Abbreviations: Df – degrees of freedom

Clearing significantly impacted on native vegetation-related variables both at 3 and 6 months after treatment application (Table 4.6). The presence of ryegrass had a significant effect on native vegetation-related variables only at 3 months after treatment application, while addition of fertilizer had no effect (Table 4.6). However, fertilizer addition significantly affected exotic plant cover at 3, but not 6 months after treatment application (Table 4.6). The site always had a significant effect on soil properties (Table 4.6); however, due to the focus of the current study on treatment effects, this was not investigated further.
Figure 4.4. Effect of experimental treatments on soil properties and vegetation at 3 months after application, detected by partial distance-based redundancy analysis. Weighted averages scores of the samples (scaling 1) (A, C) and of the original variables (scaling 2) (B, D) for the first three canonical axes (CAPs) are shown. Scaling 1 approximates the Euclidean distances between the samples in the multidimensional space, while the angles between the variables in scaling 2 reflect their correlations. Experimental treatments are coded by symbols: □ - Non-treated control; ○ - Cleared control; ▲ - Cleared + Fertilizer; △ - Cleared + Ryegrass; ▢ - Cleared + Fertilizer + Ryegrass. Dashed lines represent distances from the group centroids; continuous ellipses represent the values of one standard deviation from the group centroids; the percentage of the variability in the db-RDA model explained by the canonical axes is given in parentheses. Abbreviations: NS- number of native plant species, NC- percentage of ground covered by green native species, EC- percentage of ground covered by green exotic species, EH- average height of the exotic (ryegrass) cover, MEH- maximum height of ryegrass, NH- percentage of ground covered by re-growing and newly emerging native, Amp- ammonium by microplate method, UR - urease activity, AIP- alkaline phosphomonoesterase activity, AR- arylsulphatase activity, A- ammonium-N, P- phosphorus, K- potassium, NminNH4- ammonium in N-mineralization assay, NminNO3- nitrate in N-mineralization assay, M- moisture, ga - respiration in response to D-galacturonic acid, t- respiration in response to thiamine, C- respiration in response to cinnamic acid, k- respiration in response to a-ketoglutaric acid.
Figure 4.5. Effects of experimental treatments at 6 months after application on soil properties and vegetation detected by partial distance-based redundancy analysis. Weighted averages scores of the samples (scaling 1) (A, C) and of the original variables (scaling 2) (B, D) for the first three canonical axes (CAP) are shown. Scaling 1 approximates the Euclidean distances between the samples in the multidimensional space, while the angles between the variables in scaling 2 reflect their correlations.

Experimental treatments are coded by symbols: □ - Non-treated control; □- Cleared control; ▲- Cleared + Fertilizer; ▲- Cleared + Ryegrass; ▲- Cleared + Fertilizer+ Ryegrass. Dashed lines represent convex hulls and distances from the group centroids; continuous ellipses represent the values of one standard deviation from the group centroids; the percentage of the variability in the RDA model explained by the canonical axes is given in parentheses. Abbreviations: NC- percentage of ground covered by green native species, EC- percentage of ground covered by green exotic species, EH- average height of the exotic (ryegrass) cover, MEH- maximum height of ryegrass, NH- percentage of ground covered by re-growing and newly emerging native, Amp- ammonium by microplate method, A- ammonium-N, NO3- nitrate in N-mineralization assay, AR- arylsulphatase activity, BG- β-glucosidase activity, DE- deaminase activity, M- moisture, P- phosphorus, S- sulphur, Cond- conductivity, pH- pH in CaCl2; respiration in response to: t- thiamine, c- cinnamic acid, w- water, i- imidazole, g- D-glucose, sa- succinic acid.
**Figure 4.6.** Effects of the application of fertilizer on soil biochemical properties at 6 months after treatment application. Means of weighted averages scores of CAP1 (in scaling 1) for the relevant experimental treatments (top panel) and weighted averages scores (in scaling 2) for the original variables included in the model (bottom panel) are given. The percentage of the variability in the db-RDA model explained by the canonical axis is given in parentheses; error bars denote standard deviation of the mean. Abbreviations: Nmin-nitrogen mineralization potential, NminNO3- nitrate in N-mineralization assay, pH- pH in CaCl2, M- moisture, Cond - conductivity, OrgC- organic carbon; activity of: UR urease, AR- arylsulphatase, DE- deaminase, BG- β-glucosidase, AIP- alkaline phosphomonoesterase; (AIP); respiration in response to: g- D-glucose, ga- D-galacturonic acid, sa- succinic acid, k- α-ketoglutaric acid, i – imidazole, t- thiamine, c- cinnamic acid.
Table 4.6. Effects of experimental treatments on soil properties and vegetation at 3 and 6 months after treatment detected by variation partitioning. Effect of clearing was investigated in a one-way model with non-treated plots as control; fertilizer and ryegrass were investigated in a two-way model (*). Bold characters highlight treatment partitions corrected for all other factors; partitions not corrected for other factors are not reported (nr).

<table>
<thead>
<tr>
<th>Months after treatment application</th>
<th>Response</th>
<th>Factor</th>
<th>Clearing with litter removal</th>
<th>Treatment Fertilizer (500 kg/ha)</th>
<th>Ryegrass (0.5 kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Df</td>
<td>Adj. R²</td>
<td>pseudo-F</td>
<td>p value</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Soil properties*</td>
<td>Trt+Site</td>
<td>3</td>
<td>14</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trt-Site</td>
<td>1</td>
<td>14</td>
<td>-0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site-Trt</td>
<td>2</td>
<td>14</td>
<td>0.15</td>
</tr>
<tr>
<td>6</td>
<td>Native vegetation</td>
<td>Trt+Site</td>
<td>3</td>
<td>14</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trt-Site</td>
<td>1</td>
<td>14</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site-Trt</td>
<td>2</td>
<td>14</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Ryegrass</td>
<td>Trt+Site</td>
<td>3</td>
<td>14</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trt-Site</td>
<td>1</td>
<td>14</td>
<td>-0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site-Trt</td>
<td>2</td>
<td>14</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Soil properties*</td>
<td>Trt+Site</td>
<td>3</td>
<td>14</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trt-Site</td>
<td>1</td>
<td>14</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site-Trt</td>
<td>2</td>
<td>14</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Native vegetation</td>
<td>Trt+Site</td>
<td>3</td>
<td>14</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trt-Site</td>
<td>1</td>
<td>14</td>
<td>0.57</td>
</tr>
</tbody>
</table>

* - excluding A, Amp, K, P, NO3, S. Abbreviations: M – model; R– residual; Adj. – adjusted; na – not available; Trt - treatment.
4.4. Discussion

The current study revealed that soil biochemical properties changed as a result of fertilizer application, but not through clearing of the vegetation or incursion of exotic weed ryegrass. Unexpectedly, soil biochemical properties were not affected by experimental treatments. One explanation for this could be that microbial activity in the soils is linked to soil nitrate availability (Wu et al. 2011, Macdonald et al. 2009), and in the current study no changes in soil nitrate levels were detected when tested using conventional methods. Another explanation is that inherent soil physical properties determine soil chemical and biochemical properties (Chapter 3) to a larger extent than the experimental treatments tested in the current study. Also, differences in biochemical soil properties other than N, P, K and S contents were apparent only at 6 months after treatment, suggesting that the short duration of the experiment could have been an important limitation.

4.4.1. The effects of ryegrass and fertilization on native vegetation

The presence of ryegrass limited the recovery of the native understorey. In the short term, fertilizer and ryegrass lowered the number of native plant species recorded in the plots; however, after 6 months, none of the experimental treatments significantly influenced recovery. This could be explained by the lessening of the fertilizer effect, and related growth of ryegrass, with time, which could in turn facilitate the return of the number of native plant species to initial values after the N source was exhausted or leached. This suggests that the loss of plant diversity, when halted early, may not necessarily be long-lasting or difficult to reverse.

Ideally, rehabilitation techniques would simultaneously exclude exotic grasses and re-direct the flow of soil nutrients into native vegetation, as some of the native shrubs and trees can benefit from an increased load of certain nutrients (K. Ruthroff, pers. comm.). In the present study, the application of fertilizer did not markedly improve the recovery of the native vegetation and was associated with lower number of native
plant species at 3 months after treatment application; however, at 6 months, it was associated with increased NS in the absence of ryegrass, and decreased number of native plant species in the presence of ryegrass. This suggests that adding fertilizer may benefit native vegetation and support the re-appearance of native species after clearing, providing the exotic species, which are likely to quickly use the available surplus N and water, are absent.

4.4.2. Relationships between soil biochemical properties and ryegrass persistence

Ryegrass established successfully in both fertilized and non-fertilized plots; however, unexpectedly, a dramatic decrease in ryegrass cover, but not its height, was measured between 3 and 6 months after treatment application. This decrease tended to be smaller in the fertilized plots, suggesting that one of the possible explanations for this result could be the exhaustion of soil inorganic nitrogen stores, as ryegrass growth is usually N-limited (Andrews et al. 2011). At 6 months after treatment application, both fertilized and non-fertilized ryegrass-seeded plots had lower levels of soil ammonium and nitrate than non-treated plots. Plants can utilise N from organic sources (Warren 2006, Schimel and Bennett 2004); however, this involves processes dependent on soil microbes (Schimel and Bennett 2004), therefore alterations in soil microbial communities may affect the availability of the different forms of N. Incorporating N-fixing white clover was found to be an efficient way of improving ryegrass pasture by increasing N input, which under certain conditions was equivalent to supplying inorganic fertilizer (Andrews et al. 2011). The N-ryegrass response supports the nutrient enrichment link as has been made elsewhere, including for the remnants in this region (Archibald et al. 2011). This relationship is important because it allows for the dominance of exotic over native plant species to occur. Alteration of N cycling in the soil through favouring nitrifying microorganisms, which increase the availability of inorganic nitrate, can persist even
after the initial disturbance has ceased (Prober and Lunt 2009, Malcolm et al. 2008, Grierson and Adams 2000). Such alteration can make the ecosystem vulnerable in the longer term to the invasion of exotic weeds even after revegetation, and potentially negatively impact on the survival of planted seedlings (Davidson et al. 2007). The current study suggested that soil nitrate concentration, but not P and S, is an important factor in the process of degradation of the remnants in the study area, through maintenance of exotic species in the understorey. If the persistence of ryegrass in native soil depends on the availability of additional N sources like artificial fertilizer, livestock waste or N-fixing legumes, then the success of the restoration approaches in native vegetation would be proportional to the reduction of additional input, or of the availability of N. Mulching with organic material with high C:N ratio (e.g. sawdust, tree bark, dry leaf litter) could be one of the rehabilitation methods used to achieve this goal (Homyak et al. 2008, Malcolm et al. 2008, Blumenthal et al. 2003). Another way could be to decrease nitrification by applying a nitrification inhibitor, such as dicyandiamide (Andrews et al. 2011).

4.4.3. The effects of fertilizer on soil properties

The application of fertilizer influences the enzymatic activity of soil, most notably that of urease, where a negative relationship between the activity of this enzyme and soil ammonium content was measured soon after application. This persisted even after the initial increase in ammonium, which is the product of urease activity, but was not so prominent at 6 months after application. A decrease in urease activity was detected in soils after land use transition from forest to agricultural land in Spain (Zornoza et al. 2009). Ajwa et al. (1999) also reported that N-fertilizer increased the activity of β-glucosidase and decreased that of urease in soil of a grassland ecosystem in North America. Despite the differences in environments, the outcome in the study presented here was similar, linking the activity of soil urease and β-glucosidase to the availability of
soil ammonium and the presence of easily decomposable plant material such as herbaceous biomass.

Increased levels of P, persisting in the soil for up to 6 months following application of fertilizer, were associated with decreased soil phosphomonoesterase and arylsulphatase activities. This was not unexpected, as this nutrient is less mobile than ammonium and nitrate and the activity of the enzymes that partake in providing it in plant-available forms can decrease when their products are easily available in the soil (Nannipieri et al. 2011, Olander and Vitousek 2000). Fungal communities were shown to be susceptible to excess phosphorus in Western Australian soils, with adverse effects on native mycorrhiza-dependent plants and seedlings (Standish et al. 2007). Furthermore, the rate of decrease of plant-available P in soil may be very slow and site-dependent, as increased plant-available P levels were still found in old field soils even after a single year of cultivation and 45 years of recovery (Standish et al. 2006). Such persistent changes in soil nutrient pools could potentially contribute to the poor success of native seedling establishment in degraded remnants during restoration attempts, by seriously impacting microbial life that is of importance to plant survival in naturally oligotrophic environments (Standish et al. 2007).

4.4.4. The effects of clearing

Exotic plant species did not establish due to clearing alone; however, some germination of exotic plant species (totalling less than 0.5% cover of the plot area) was found in cleared plots that were fertilized, but not seeded. The presence of exotic grasses at site Mi, albeit over 20 m away from the experimental plots, suggested that exotics may be present in the soil seed bank or are easily imported from off-site and that nutrients are the most significant impediment to weed proliferation at this site. This would imply that clearing and fertilization would have a negative impact on the site in terms of facilitating weed invasion but only if the exotic species were already present or nearby.
Clearing of native vegetation resulted in increased numbers of *Eucalyptus* spp. seedlings (data not shown), which was not unexpected, as many *Eucalyptus* species benefit from decreased competition after the removal of neighbouring vegetation (Yates *et al.* 2000b).

### 4.4.5. Biochemical soil properties related to native vegetation regrowth after disturbance

The potential to support weed growth and to regenerate the native plant community was noticeably site-dependent. When soil N is repeatedly supplied by regular artificial fertilizers and exotic grass becomes firmly established, as happens on a large scale in agriculture-dominated landscapes in the SWWA (Yates and Hobbs 1997, Yeates 1993), the alterations in nutrient cycling become a serious obstacle to restoration (Archibald *et al.* 2011, Davidson *et al.* 2007, Prober and Thiele 2005). Therefore, if remnant vegetation has already been highly modified with low soil pH, high P concentration and the presence of exotic grasses, the potential for recovery following the removal of degrading factors may be low, or non-existent. Indeed, the most disturbed site in this study (Mi) had poorer plant growth, both native and exotic ground cover, and the poorest recovery of the number of native plant species recorded in plots of all three sites, even though the original native cover was not the lowest. This result supports the concept of restoration thresholds of biotic and abiotic origin (Hobbs and Harris 2001, Hobbs and Norton 1996), which prevent the ecosystem from returning to the pre-disturbance state without the input of restoration effort.

### 4.5. Conclusions

Excess soil nutrients had greater influence on soil biochemical properties than other types of disturbance. The presence of exotic weeds, such as ryegrass, depended on the availability of soil nitrate, but not P or S content, and negatively impacted the regrowth of native vegetation. However, over the 6 month period of the study, the presence of ryegrass did not alter soil biochemical properties.
Chapter 5. The effects of restoration treatments on soil biochemistry and the survival and growth of planted seedlings

5.1. Introduction

In the south west of Western Australia native forest remnants in *E. globulus* plantations are a source of biodiversity, and as such forest certification schemes expect plantation companies to manage them for conservation. Remnants with native understorey harbour more biodiversity, have higher rates of native tree recruitment and a higher proportion of seed-bearing and live trees than those with exotic understorey (Archibald et al. 2011). Consequently, rehabilitation of the remnants in which native understorey has been replaced with exotic plant species may increase the chances of maintaining the biodiversity within plantation estates (Archibald et al. 2011). However, land managers often have to resort to a “trial and error” approach in order to choose the best restoration method. Then they are faced with uncertainty as to how to measure the success of their management interventions, particularly over the short to medium term. This uncertainty is partly due to lack of relevant research into restoration methods and practices for specific plant communities, as well as of consensus in restoration ecology as to the best indicators of biodiversity or restoration success (Brudvig et al. 2011, Ruis-Jaen and Aide 2005).

Restoration of remnant native vegetation has been studied in Australia in the context of agricultural landscapes (Archibald et al. 2011, Prober and Lunt 2009, Close et al. 2008, Yates et al. 2000a). Suggested methods and practices for restoration include elimination of exotic plant species, exclusion of grazers, removal of topsoil to limit nutrient excess and restoration plantings. Yates et al. (2000a) reported that reducing weed load by using herbicides (glyphosate with simazine) prior to revegetation improved the establishment of native woody perennials and trees in remnants of *E. salmonophloia* woodland in the Wheatbelt region of WA. The role of increased soil nitrate in maintaining
the exotic plant species in native landscapes (Chapter 4, Malcolm et al. 2008) and the responsiveness of soil microbial community to this nutrient (Macdonald et al. 2009, Kasel et al. 2008) suggests that microbial activity in the soil may play pivotal role in supporting or impeding restoration. Mulching with organic residue can successfully reduce weed cover in forests and in agricultural systems by physically disrupting the growth of weeds (Teasdale and Mohler 2009), increasing retention of soil moisture (Lambert et al. 1994) and altering soil nutrient pools by changing C: N ratios and stimulating microbial activity (Blumenthal 2009, Prober and Lunt 2009, Homyak et al. 2008, Blumenthal et al. 2003). In addition, beneficial effects of mulching on germination and establishment of seedlings of several plant species indigenous to WA have been reported (Grose 2011). None of the above-mentioned restoration methods has been systematically studied in remnant vegetation within *E. globulus* plantations in WA.

Microplate-format assays for determining soil biochemical properties were useful in discerning between land condition, including remnants with exotic and native understorey (Chapter 3), and were used to detect changes in soils due to fertilizer application in an experiment done on soil with similar structure (Chapter 4). Moreover, the results presented in Chapter 4 suggest that soil nitrate content is an important determinant for the persistence of exotic grasses in remnants with native understorey and that soil chemical and biochemical properties may be useful in determining the susceptibility of a site to degradation. An analogous analysis of soil properties and the survival and growth of the planted seedlings would be useful to determine indicators of revegetation outcomes. Presence of such relationships would provide preliminary proof of the usefulness of the soil properties as a diagnostic tool for investigating restoration methods and choosing plant species for re-planting protocols.

To provide plantation managers with practical guidelines for rehabilitation of remnant vegetation, the effects of selected restoration treatments on soil biochemical characteristics and on the success of planted seedlings of native shrub and tree species
were investigated during two year-long field trials conducted in the SWWA. Mulching with plantation harvest residue and/or application of herbicides (glyphosate) were chosen as restoration treatments for their proven effectiveness in other systems (Grose 2011, Yates et al. 2000a), and for their practical merits, such as availability and ease of application. Covering with black plastic was chosen as an alternative to mulching for weed suppression (Lambert et al. 1994; Truax and Gagnon 1993). A pilot study was conducted to pre-screen the potential restoration treatments and plant species used for replanting (Appendix 1). From the pilot study it was determined that herbicides application and mulching with plantation harvest residue had an effect on the growth and survival of the seedlings; however, no effects of the restoration treatments on soil biochemical properties were detected. Consequently, to validate this unexpected result, in the current study the growth and survival of planted seedlings, exotic ground cover and selected soil biochemical and chemical properties were measured to test the following hypotheses:

(i) application of herbicides and mulching increase native seedling growth
(ii) mulching with organic material reduces soil nitrate content
(iii) mulching with organic material alters the biochemical properties of the soil as detected by microplate-format assays.

5.2. Materials and methods

5.2.1. Experimental design and sampling procedures

Three remnants with similar exotic understorey composition were selected in three 7-10 years old *E. globulus* plantation estates, located within a 30 km radius of each other (Ma, P and S, see Fig. 2.1, Chapter 2). Selected characteristics of the soils from these sites are given in Table 5.1. In each remnant, 12 experimental plots (4 x 4 m) were established under similar canopy cover (<10%). No tree stems were present within the plots and the ground cover was uniform (80-100%), comprising mostly of exotic grasses,
except for site P, where *Trifolium* spp. was also present. Three replicate plots were allocated to each of the four experimental treatments in each of the remnants, and the following treatments were applied in November 2009, at the beginning of the dry season (summer): (1) non-treated control; (2) glyphosate, a broad-spectrum herbicide with little or no effects on soil microorganisms in the field conditions (Weidenhamer and Callaway 2010, Busse et al. 2001) as 1% v/v solution of Roundup™ (Monsanto Co., Columbus, OH) at 0.2 l m$^{-2}$, equivalent to 0.72 g m$^{-2}$ glyphosate; (3) mulch: an even, approximately 10 cm thick layer of finely shredded *E. globulus* harvest residue produced using an infill chipper; and (4) black construction plastic of 0.2 mm thickness (Table 5.2). The treatments were left undisturbed over the summer. Six months after treatment application, at the onset of wet season in May the following year, the plastic was removed. Eight months after treatment application in July, 6 week-old seedlings of three native plant species: *Eucalyptus marginata* (ja), *Agonis flexuosa* (ag) and *Acacia saligna* (ac) (8, 11 and 16 seedlings per plot, respectively) were planted in all plots in a 6 x 6 grid with regular 30 cm spacing between seedlings, whenever possible. The plots were fenced-off to minimize the impact of large herbivores. Another round of glyphosate application, using the protocol described above, was sprayed on glyphosate and mulch-treated plots just before planting of seedlings. Survival (s), average height (ah) and maximum height (mh) was monitored for each species. The percentage of plot area covered by exotic plants (EC): grasses (gr) and broadleaf weeds (br), was assessed by two independent observers at 3 and 10 months after planting.

Soil sampling was done before treatment application for baseline measurements, and at 8 and 11 months after treatment application, i.e. prior to and 3 months after planting. Eight cores of 5 cm diameter and depth were collected from each plot using a sampling grid that ensured a unique location was sampled each time and thereby avoiding the sampling of soils disturbed by the previous collection. Samples were
transported to the laboratory in sealed plastic bags at ambient temperature and processed within 4 days from collection.

Table 5.1. Mean values ±SD of selected chemical properties of the soil in the study sites.

<table>
<thead>
<tr>
<th>Soil property (abbreviation)</th>
<th>Unit</th>
<th>Reference</th>
<th>Site</th>
<th>Site</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ma</td>
<td>P</td>
<td>S</td>
</tr>
<tr>
<td>Gravimetric moisture (M)</td>
<td>%</td>
<td>Buurman et al. (1996)</td>
<td>25.86 ± 5.00</td>
<td>21.52 ± 4.49</td>
<td>29.97 ± 4.45</td>
</tr>
<tr>
<td>Nitrate-N (NitN)</td>
<td>mg kg⁻¹</td>
<td>Searle (1984)</td>
<td>28.83 ± 16.08</td>
<td>21.57 ± 7.04</td>
<td>49.75 ± 26.84</td>
</tr>
<tr>
<td>Ammonium-N (A)</td>
<td>mg kg⁻¹</td>
<td>Searle (1984)</td>
<td>11.25 ± 3.74</td>
<td>9.75 ± 5.38</td>
<td>8.83 ± 11.41</td>
</tr>
<tr>
<td>Exchangeable phosphorus - Colwell (P)</td>
<td>mg kg⁻¹</td>
<td>Rayment and Higginson (1992)</td>
<td>28.08 ± 4.74</td>
<td>22.08 ± 7.91</td>
<td>24.00 ± 6.70</td>
</tr>
<tr>
<td>Exchangeable potassium - Colwell (K)</td>
<td>mg kg⁻¹</td>
<td>Rayment and Higginson (1992)</td>
<td>214.25 ± 98.28</td>
<td>106.5 ± 73.17</td>
<td>104.50 ± 55.66</td>
</tr>
<tr>
<td>Sulphur (S)</td>
<td>mg kg⁻¹</td>
<td>Blair et al. (1991)</td>
<td>6.81 ± 4.18</td>
<td>5.86 ± 2.56</td>
<td>5.85 ± 1.41</td>
</tr>
<tr>
<td>Organic carbon (OrgC)</td>
<td>%</td>
<td>Walkley and Black (1934)</td>
<td>8.96 ± 0.00</td>
<td>6.86 ± 0.68</td>
<td>6.34 ± 1.12</td>
</tr>
<tr>
<td>Conductivity (Cond)</td>
<td>dS m⁻¹</td>
<td>Rayment and Higginson (1992)</td>
<td>0.11 ± 0.04</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>pH in CaCl₂ (pH)</td>
<td>-</td>
<td>Rayment and Higginson (1992)</td>
<td>4.78 ± 0.15</td>
<td>4.48 ± 0.21</td>
<td>4.23 ± 0.21</td>
</tr>
<tr>
<td>Total nitrogen – Kjedahl (TotN)</td>
<td>%</td>
<td>Rayment and Lyons (2011)</td>
<td>0.93 ± 0.21</td>
<td>0.48 ± 0.09</td>
<td>0.44 ± 0.10</td>
</tr>
</tbody>
</table>

5.2.2. Laboratory analyses

The activity of soil alkaline phosphomonoesterase (AIP), urease (UR), arylsulphatase (AR), deaminase (DE), β-glucosidase (BG) was tested according to the Protocol 1 (Chapter 2.2.6). Soil chemical properties were determined as described in Chapter 2.2.3. Soil respiration induced by water (w) and substrates: D-glucose (g), D-galacturonic acid (ga), succinic acid (sa), α-ketoglutaric acid (k), imidazole (i), thiamine (t) and cinnamic acid (c) was assayed as described in Chapter 2.2.5. Soil nitrogen mineralization potential assay (NminNH₄ and NminNO₃) and ammonium (Amp) and nitrate (NO₃) in soil extracts was measured in microplate-format as described in Chapter 2.2.4.
Table 5.2. Experimental treatments in the study investigating selected restoration practices in the remnants of native vegetation in *Eucalyptus globulus* plantations in the south west of Western Australia.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glyphosate 0.72 g m$^{-2}$</th>
<th>Harvest residue</th>
<th>Black plastic</th>
<th>Replicate plots per site</th>
<th>Planted plots per site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-treated control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Mulch</td>
<td>-</td>
<td>√</td>
<td>-</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Plastic</td>
<td>-</td>
<td>-</td>
<td>√</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

5.2.3. **Statistical analyses**

The data were pre-treated as described in Chapter 2.3. Principal component analysis (PCA) using standardized data from the baseline data set were used to investigate the relationships among the original variables. The effects of treatment and site were analyzed using variation partitioning and distance-based redundancy analysis as described in Chapter 2.3. For the initial, baseline data set, testing for the effects of treatment as a dummy factor was used to investigate the background differences among experimental plots that could confound the results. Only the models with significant effects of the experimental treatments are reported in detail.

The effects of site and treatment were tested on soil properties data before and at 8 and 11 months after treatment application, and on exotic groundcover measures with seedlings survival and growth data at 3 and 10 months after planting, i.e. at 11 and 18 months after treatment application. All groups had $n = 9$ replicates.

In addition, univariate analysis of variance (ANOVA) was done using the exotic ground cover and seedlings survival and growth data. The exotic ground cover measures and seedling growth and survival were tested for restoration treatment and site effects using fixed-factor two-way ANOVA. The quality of the models was determined by
inspection of the residual plots and Tukey’s Honestly Significant Difference tests were used to correct for multiple comparisons.

5.3. Results

5.3.1. The effects of treatment on soil biochemistry, exotic ground cover and seedlings growth and survival

There were no differences in soil properties among plots before treatment application (Table 5.3). However, there was a significant effect of site on soil properties (Table 5.3), which was also evident in the PCA (Fig. 5.1). The soils from different sites varied especially in N-related properties such as total N, ammonium and nitrate content and N-mineralization potential, and enzymatic activity (Fig. 5.1). The appearance of the experimental plots after application of treatments (Figs. 5.2 and 5.3) was similar within treatments across the sites, with non-treated control plots similar to the areas surrounding the experimental plots.

Significant effects of the experimental treatments on soil properties were measured only at 11 months after application, while the effects on exotic ground cover and seedlings survival and growth were significant at both 3 and 10 months after planting (Table 5.3). All treatments significantly reduced the overall exotic ground cover prior to planting. Mulching was as effective as glyphosate treatment; however, the plastic treatment had the strongest effect (Fig. 5.4 A). At 3 months after planting, the glyphosate and mulch treatments were most effective at reducing exotic grass cover with plastic treatment being less effective (Fig. 5.4 B). At 10 months after planting, the effect of the plastic treatment was no different from the control, while both glyphosate application and mulching reduced exotic ground cover. The difference between these two latter treatments was not significant (Fig. 5.4 C).
Table 5.3. Effects of experimental treatments on soil properties, exotic ground cover and survival and growth of planted seedlings, detected by variation partitioning. Models focusing on the effects of experimental treatments independent of other factors are highlighted in bold.

<table>
<thead>
<tr>
<th>Response</th>
<th>Month</th>
<th>Factor</th>
<th>Df</th>
<th>Adj. $R^2$</th>
<th>Pseudo-F</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mod</td>
<td>Resid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil properties</td>
<td>0</td>
<td>Treatment*+Site</td>
<td>5</td>
<td>30</td>
<td>0.17</td>
<td>2.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment* - Site</td>
<td>3</td>
<td>30</td>
<td>-0.01</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site - Treatment*</td>
<td>2</td>
<td>30</td>
<td>0.2</td>
<td>4.81</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Treatment + Site</td>
<td>5</td>
<td>30</td>
<td>0.2</td>
<td>2.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment - Site</td>
<td>3</td>
<td>30</td>
<td>0.01</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site - Treatment</td>
<td>2</td>
<td>30</td>
<td>0.22</td>
<td>5.36</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Treatment + Site</td>
<td>5</td>
<td>30</td>
<td>0.26</td>
<td>3.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment - Site</td>
<td>3</td>
<td>30</td>
<td>0.02</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site - Treatment</td>
<td>2</td>
<td>30</td>
<td>0.26</td>
<td>6.54</td>
</tr>
<tr>
<td>Exotic ground cover and seedling survival and growth</td>
<td>11/3</td>
<td>Treatment + Site</td>
<td>5</td>
<td>30</td>
<td>0.19</td>
<td>2.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment - Site</td>
<td>3</td>
<td>30</td>
<td>0.14</td>
<td>2.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site - Treatments</td>
<td>2</td>
<td>30</td>
<td>0.07</td>
<td>2.46</td>
</tr>
<tr>
<td>Exotic ground cover and seedling survival and growth</td>
<td>18/10</td>
<td>Treatment + Site</td>
<td>5</td>
<td>30</td>
<td>0.29</td>
<td>3.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment - Site</td>
<td>3</td>
<td>30</td>
<td>0.12</td>
<td>2.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site - Treatment</td>
<td>2</td>
<td>30</td>
<td>0.19</td>
<td>5.28</td>
</tr>
</tbody>
</table>

* - dummy factor; a-months after treatment application for soil properties and exotic ground cover / months after planting for seedlings survival and growth data. Abbreviations: Df – degrees of freedom; Mod – model; Resid – residual; Adj. – adjusted.
Figure 5.1. First two principal components of the analysis of soil properties before application of restoration treatments. The percentage of the variability in the data set explained by the principal components is given in parentheses. Symbols code for sites: □ = Ma; ○ = P; ▲ = S. Lines represent component loadings. Abbreviations: activity of: alkaline phosphomonoesterase (AlP), urease (UR), arylsulphatase (AR), deaminase (DE), β-glucosidase (BG); respiration in response to: water (w), D-glucose (g), D-galacturonic acid (ga), succinic acid (sa), α-ketoglutaric acid (k), imidazole (i), thiamine (t), cinnamic acid (c); ammonium by microplate method (Amp), nitrate by microplate method (NO3), nitrate in N-mineralization assay (NminNO3), N-mineralization (NminNH4), nitrate-N (NitN), ammonium-N (A), phosphorus (P), potassium (K), sulphur (S), organic carbon (OrgC), total nitrogen (TotN), conductivity (Cond), pH in CaCl₂ (pH), moisture (M).
Figure 5.2. Effects of selected restoration treatments in site S: covering with black plastic (A, B) and mulching with harvest residue (C, D) immediately (A, C) and 8 months after (B, D) application. Glyphosate-treated plots were similar to (B) at this time.
**Figure 5.3.** Effects of restoration treatments in site P: glyphosate (A); covering with black plastic (B) and mulching with harvest residue (C). Representative plots at 11 months after application are shown.
Covering the plot with black plastic significantly reduced both exotic grass and broadleaf plant cover before planting, with mulch having an effect only on broadleaf plants (Fig. 5.4 A). At 3 months after planting, all treatments significantly reduced both grass and broadleaf ground cover when compared to control plots, with glyphosate and mulch treatments having the strongest effect (Fig. 5.4 B). At 10 months after planting, the glyphosate and mulch treatments significantly altered exotic ground cover (Fig. 5.4 C). Percentage of plot surface covered by broadleaf plants was not altered by any of the treatments; however, the exotic grass cover was significantly reduced by glyphosate and mulch, with stronger effect of mulch (Fig. 5.4 C).

The black plastic treatment had no effect on seedling survival and there was no interaction between this treatment and site apparent in the ANOVA models (data not shown). At 3 months after planting, all of the rehabilitation treatments increased the survival of *A. saligna*, with similar patterns found for other species, which were not significant (Fig. 5.5 A). By 10 months after planting, the improvement in survival as compared to control was over 15% for *A. flexuosa* and over 30% for *E. marginata* and *A. saligna* (Fig. 5.5 B). Glyphosate and mulch treatments equally and significantly increased the survival of the seedlings of all three native plant species at 10 months after planting (Fig. 5.5 B).
Figure 5.4. Effects of rehabilitation treatments on the percentage ground cover of exotic grass and broadleaf plants (A) before planting/at 8 months after treatment application, (B) 3 months, and (C) 10 months after planting (at 11 and 18 months after treatment application, respectively). The p values for differences from the respective non-treated controls are given next to the bars: * p < 0.05; ** p < 0.01; *** p < 0.001. Different letters denote differences among treatments in percentage ground cover as the sum of exotic grass cover (%) and broadleaf plant cover (%). Error bars show one standard deviation of the mean.
Table 5.4. Significance of the canonical axes (CAPs) in the distance-based redundancy analysis of the effects of experimental treatments on soil properties, exotic ground cover and survival and growth of planted seedlings. Bold characters highlight values significant at the level of $\alpha = 0.05$.

<table>
<thead>
<tr>
<th>CAP</th>
<th>Soil properties</th>
<th>Exotic ground cover and seedling survival and growth</th>
<th>Exotic ground cover and seedling survival and growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11/3</td>
<td>18/10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Df</td>
<td>Var</td>
<td>$F^*$</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0.006</td>
<td>1.87</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.004</td>
<td>1.16</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0.003</td>
<td>1.06</td>
</tr>
<tr>
<td>4</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Residual</td>
<td>30</td>
<td>0.095</td>
<td>30</td>
</tr>
</tbody>
</table>

- at 10 months after planting, data from planted plots only; b - at 11 months after treatment application, i.e. 3 months after planting; c – at 10 months after planting; $F^*$- pseudo-F. Abbreviations: Df – degrees of freedom; Var- variance; na- not available.
There were no differences in maximum and average seedling height in all treatments as compared to control plots at 3 months after planting (Fig. 5.6 A and B). However, at 10 months both glyphosate and mulch treatments significantly increased both these variables for *A. flexuosa* and *A. saligna* (Fig. 5.6 C and D). Mulching increased maximum height of jarrah seedlings and the black plastic treatment increased average height of *A. saligna* (Fig. 5.6 C and D).

**Figure 5.5.** Effect of rehabilitation treatments on the percentage survival of the seedlings of the three native plans species at (A) 3 and (B) 10 months after planting. Error bars denote standard deviation of the mean (n = 9); p values for differences from the respective controls are given as: * p <0.05; ** p <0.01; *** p <0.001.
Figure 5.6. Effects of various rehabilitation treatments on maximum and average height of the seedlings of the three native plant species at (A, B) 3 months and (C, D) 10 months after planting. Error bars denote standard deviation of the mean (n = 9); p values for differences from the respective controls are given as: * p <0.05; ** p <0.01; ***p <0.001.
A clear difference in soil properties between mulched and control plots was apparent at 11 months after treatment application (corresponding to 3 months after planting). This was evident from the first canonical axis (CAP1) (Fig. 5.7 A) which was the only one explaining a sufficiently large proportion of the variation to be interpreted with confidence (Table 5.4). Mulching increased soil moisture, nitrate, P and K concentration in soil and enzymatic activity as compared to control plots, while N-mineralization potential and respiration in response to thiamine and imidazole decreased (Fig. 5.7 B).

The db-RDA of the exotic ground cover and seedlings survival and growth data both at three and at 10 months after planting resulted in the generation of three canonical axes of which only the first CAP in each case explained a substantial proportion of the total variation and could be interpreted (Table 5.4). At both times, the difference between the control and glyphosate, as well as mulch-treated plots was evident along these (Fig. 5.8 A and C). The exotic species cover was reduced, and seedling survival was increased in plots treated with mulch and glyphosate, as compared to control (Fig. 5.8).

5.4. Discussion

Restoration treatments tested in the two short-term field trials described here and in the Appendix I had different effects on the survival and growth of the native seedlings used for revegetation of the remnants in *E. globulus* plantations and on the cover of exotic species. The hypotheses relating to reduction of exotic ground cover and increased seedling survival and growth as a consequence of the application of herbicides and mulching were supported.
**Figure 5.7.** Effects of the experimental treatments on soil properties at 11 months after treatment application, detected by partial distance-based redundancy analysis. Weighted average scores of the samples (scaling 1) (A) and of the original variables (scaling 2) (B) for the first two canonical axes (CAPs) are shown. Scaling 1 approximates the Euclidean distances between the samples in the multidimensional space, while the angles between the variables in scaling 2 reflect their correlations. Experimental treatments are coded by symbols: ○ - Non-treated control; △ - Mulching with harvest residue; ● - Glyphosate; □ - Plastic. Dashed lines represent distances from the group centroids; continuous ellipses represent the values of one standard deviation from the group centroids; the percentage of the variability in the db-RDA model explained by the CAPs is given in parentheses. Abbreviations: activity of: alkaline phosphomonoesterase (AIP), urease (UR), arylsulphatase (AR), deaminase (DE), β-glucosidase (BG); respiration in response to: water (w), D-glucose (g), D-galacturonic acid (ga), succinic acid (sa), α-ketoglutaric acid (k), imidazole (i), thiamine (t), cinnamic acid (c); ammonium by microplate method (Amp), nitrate by microplate method (NO3), nitrate in N-mineralization assay (NminNO3), N-mineralization (NminNH4), ammonium-N (A), nitrate-N (NitN), phosphorus (P), potassium (K), sulphur (S), organic carbon (OrgC), total nitrogen (TotN), conductivity (Cond), pH in CaCl$_2$ (pH), moisture (M).
The effects of treatment on soil properties were detected in the current study, where there was a clear difference between the control and addition of mulch, but not in the trial described in Appendix I. The results of the current study provided the only evidence supporting the last hypothesis posed in the Introduction that restoration treatments alter soil biochemical properties as detected using microplate format assays. Greater soil moisture levels recorded in mulched treatments were probably the main driver of increased enzymatic activity in these soils. However, higher soil moisture content was not associated with increased soil respiration, and there was an inverse relationship between soil moisture content and respiration in response to imidazole, as was also found in experiments reported in Chapters 3 and 4. This suggested that soil catabolic potential is influenced more strongly by other factors. Changes in soil nutrient content caused by the treatments may have also contributed to the observed differences.

5.4.1. The effects of mulch

Mulching with plantation harvest residue decreased exotic ground cover to the same extent as application of herbicide, while providing equally good or greater enhancement of the growth and survival of native seedlings. These beneficial effects could be mediated by “locking-up” excess soil nutrients, achieved by mulching with organic material with high C: N ratio. Homyak et al. (2008) reported a decrease in soil ammonium and nitrate concentration following mulching with woodchips in a northern hardwood forest. There was a concomitant increase in N in the woodchip layer, which suggested that addition of material high in C: N may cause at least partial relocation of N from the soil to the mulch layer by soil microorganisms, and potentially can encourage denitrification. Moreover, Blumenthal et al. (2003) reported a significant decrease in exotic plant biomass and an increase in native plant biomass following incorporation of sawdust into prairie soil. These effects were ameliorated by addition of N, suggesting that N-immobilization initiated by the presence of
the high C: N material was the main driver of the changes (Blumenthal et al. 2003). As a result, mulching was considered likely to have a significant impact on restoration success if the exotic species were nitrophilic (Blumenthal et al. 2003).

Chapter 4 provided evidence of the dependence of the exotic ryegrass on soil nitrate, thus mulching may be beneficial for eliminating grassy understorey in remnants with exotic understorey in *E. globulus* plantations by limiting available soil nitrate. However, in the current study, mulching with plantation harvest residue actually increased soil nitrate. Therefore, the second hypothesis of the current investigation (that mulching decreases soil nitrate) was not supported. On the other hand, nitrate generation in the N-mineralization assay, which could be considered an approximation of the nitrification potential, was reduced by the mulch treatment. This suggests that when the nitrate pool increases due to mulching, the potential of the soil to generate nitrate decreases as a result of a negative feedback mechanism. This indicates the usefulness of the microplate format N-mineralization assay in investigating the processes underlying nutrient cycling.

A marked increase in K concentration in soil was measured in the mulch treatments in this study. This is in agreement with an earlier study investigating the effects of straw mulch, where changes in soil nitrate and K concentrations similar to those observed in the current study were attributed to the decomposition of organic matter and leaching of the mobilised nutrients from the mulch (Truax and Gagnon 1993). In the current study, an attempt to tackle this problem by using woody mulch was made; however, without success. A possible explanation for this could be that the nutrient flush was a result of the decomposition of the herbaceous exotic groundcover under the woody mulch, possibly facilitated by higher moisture levels.
Figure 5.8. Effect of treatments on exotic ground cover and seedling growth and survival at (A, B) 3 and (C, D) 10 months after planting, detected by partial distance-based redundancy analysis. Weighted average scores of the samples (scaling 1) (A, C) and of the original variables (scaling 2) and (B, D) for the first two canonical axes (CAPs) are shown. Treatments are coded by symbols: □- Non-treated control; △- Mulching with harvest residue; ◆- Glyphosate; ■- Plastic. The abbreviations are as given in Chapter 5.2.2. Dashed lines represent distances from the group centroids; continuous ellipses represent the values of one standard deviation from the group centroids; the percentage of the variability in the db-RDA model explained by the canonical axes is given in parentheses. Abbreviations: percentage of ground covered by grass (gr), percentage of ground covered by broadleaf exotic species (br), Agonis flexuosa seedlings: survival (sag), average height (ahag) and maximum height (mhag); E. marginata seedlings: survival (sja), average height (ahja) and maximum height (mhja); Acacia saligna seedlings: survival (sac), average height (ahac) and maximum height (mhac).
The addition of labile C in the form of sucrose or glucose was proposed as a restoration practice to decrease soil nitrate levels, limiting the growth of exotic plants and encouraging the growth of native plant species in grassland ecosystems (Blumenthal 2009, Prober and Thiele 2005, Blumenthal et al. 2003). However, the carbon source had to be reapplied regularly for any benefits to persist. Considering the costs and logistics of this practice, application of woody harvest residue is the most efficient treatment of carbon enrichment in a plantation setting, particularly as the residue is considered to be a waste by-product and can be easily obtained on site in sufficient quantities to cover small remnant patches (G. Rolland, APFL, pers. comm.).

When the exotic groundcover is dense enough, the herbaceous plants killed by herbicides can be considered to be mulch as well. In the current study, such “indirect mulching” was not as efficient as the harvest residue mulch in retaining moisture or affecting soil properties and plant growth. Moreover, the woody mulch reduced the exotic groundcover to a similar extent as the glyphosate treatment and enhanced the growth of native plants species more than the herbicide treatment. This suggests that the benefits of the mulch extend beyond eliminating weeds. One of the most likely reasons for increased seedling survival in this treatment is an increase in soil moisture. The mortality of the seedlings in restoration plantings in arid areas is highest in the first summer after planting (Ruthrof et al. 2011, Benayas et al. 2002, Castro et al. 2002). Mulch can provide additional nutrients to the growing seedlings, as well as physical protection from herbivores if it includes thicker unbroken branches and shrubs (Ludwig and Tongway 1996).

5.4.2. The effects of herbicides

Exotic plant species are one of the biggest impediments to restoration, as they compete for water and nutrients with the native seedlings. The combination of glyphosate with simazine, investigated in a study described in Appendix I, was an efficient treatment for
the reduction of both exotic grass and broadleaf plants compared to other treatments; however, it was not favourable for seedling growth and survival with the exception of *A. pulchella*. Simazine is not generally considered to be toxic to native Australian plant species and has been successfully used in restoration of *E. salmonophloia* remnants in WA (Yates *et al.* 2000a). On the other hand, unexpected sensitivity of the Australian native grass *Themeda* spp. to simazine has been reported (Cole *et al.* 2004). Toxicity of simazine to soil microorganisms has not been studied in detail except in sediments and waterways (e.g. Schaefer *et al.* 2012), and the majority of the studies on the effect of this herbicide focus on its biodegradation by soil microbes (e.g. Morgante *et al.* 2012). However, a recent report of inhibition of nitrification in fertilized agricultural soil suggested that simazine can affect soil microbial communities when soil ammonium content is elevated, although the mechanisms are unknown (Hernandez *et al.* 2011). If the toxicity of simazine to *Themeda* grass represents an indirect effect of the herbicide mediated through its effects on soil microbes and nitrogen cycling, a further investigation of the interactions between soil microorganisms, herbicides and plants is needed to elucidate the mechanisms of such mediation.

The effects of glyphosate on soil properties were not clear. Herbicides can impact soil biochemistry directly and indirectly (for discussion see for example Nannipieri *et al.* 1994). The effects of glyphosate on soil biochemical properties have been documented in numerous papers and reviewed extensively (Wiedenhamer and Callaway 2010, Grover and Cessna 1991). Sannino and Gianfreda (2001) found that glyphosate increased urease and invertase activity and decreased phosphomonoesterase activity depending on soil type. Powell *et al.* (2009) reported that glyphosate increased the ratio of fungi to bacteria in the soils. In the current study, the effects of glyphosate were probably indirect and eventuated through death of the exotic grasses, thus providing organic matter for microbial decomposition. It needs to be noted that it was not possible to establish controls for other ingredients of the commercial herbicide formulations used in the study. These unknown
substances may have effects on plants and soil microbial communities independent of the active ingredients and could have contributed to the confounding of the results.

The results of the current study suggested that the response of native seedlings to herbicides may vary from species to species. This should be investigated more thoroughly to unveil the mechanisms through which this occurs and it may facilitate the generation of treatment-plant species combinations for effective restoration protocols.

5.4.3. The effects of plastic covering

There was no significant effect of plastic covering on soil biochemical properties, which was surprising considering reports of increased soil temperature and improved growth of other plantation species using this treatment (Lambert et al. 1994). In the current study, black plastic covered the plots during a typically hot summer period to provide a physical barrier to weed growth and to increase the temperature of the soil in order to inhibit the seed bank of exotic plants. This proved to be unsuccessful, suggesting that the conditions created were not sufficient to impact on the seed bank or soil properties. On reflection, this was not entirely unexpected, considering that soils in the SWWA are regularly exposed to hot and dry conditions during summer, with soil moisture dropping below 10% in the hottest months (Grierson and Adams 2000). Exposure of soil from jarrah forest to temperatures of 60 °C for 20 min in a laboratory setting did not cause any detectable changes in soil catabolic potential, suggesting some degree of resilience of soil and soil microbial communities to elevated temperatures (Banning and Murphy 2008).

Even though covering with plastic was not an effective restoration treatment in the current study, another form of plastic mulch, i.e. woven plastic weed-mat, has been successfully used to limit the weed load in restoration of wetlands (Grose 2012). This type of covering allows moisture infiltration and provides a physical barrier rather than altering the temperature or moisture content of the soil below. Up-scaling of such a treatment in a
planted setting is problematic, as covering larger areas in between the established trees, as well as removing the non-biodegradable mulch from underneath growing seedlings is difficult (Grose 2012).

Another weed-control strategy that has been tested as a means to control exotic plant species in Australia is controlled burning (Prober and Thiele 2005). Even though fire can successfully eliminate plant biomass, its use as a restoration tool is under debate as it often promotes weed growth by providing nutrients and stimulating seed germination post-fire (Davies et al. 2000). In addition, conducting prescribed burning in a plantation setting is fraught with problems and unlikely to be implemented by plantation managers on a large scale (B. Edwards, Great Southern, pers. comm.).

5.4.4. Species-specific response to treatments

Species-specific responses to the restoration treatments were evident both in the current study and in the pilot study described in Appendix I. For example, Acacia seedlings benefited from increased soil nutrient availability and reduced competition from the weeds and were not negatively impacted by residual herbicides. Possibly for these reasons, introduced species of Acacia can be a pest in E. globulus plantations, often growing to the size that requires intervention from plantation managers (B. Edwards, pers. comm). Acacia spp. generally form symbiotic associations with N-fixing bacteria and can persist in nutrient-poor soils. The current studies suggested that to ensure successful establishment of the seedlings of species other than Acacia, mulching would be preferred over herbicide treatments.

5.5. Conclusions

Mulching with plantation harvest residue with occasional spraying with glyphosate to control exotic grasses is likely to improve the survival and growth of native plant species
used to restore the understorey of the native vegetation remnants within *E. globulus* plantations. The current study showed that mulching with plantation harvest residue effectively reduced exotic groundcover and improved the survival and growth of native plant seedlings. Mulching can usually be achieved on the scale of a whole remnant at a manageable cost, which gives the plantation managers a viable means to use by-products of plantation operations to maintain or improve the biodiversity within their estates. The usefulness of biochemical soil properties in monitoring restoration was found to be restricted to the indicators of nitrification. However, due to the small scale and short duration of the two current studies, more research would be needed to develop detailed restoration prescriptions and practical guidelines for wider use. Especially, investigation of other restoration treatments aiming to reduce excess soil nutrients, such as incorporation of biochar, could be conducted. Elucidation of species-specific responses to different restoration treatments in the survival and growth of native plant seedlings is recommended.
Chapter 6. Functional diversity and relationships among soil properties.

6.1. Introduction

Investigations of soil microbial communities and their function often rely on indirect measurements of their activity through indicators, such as soil biochemical properties (Romaniuk et al. 2011). Changes in soil other properties are also often measured, as disturbance can affect the chemical and physical characteristics of a soil, which in turn influences its biochemical properties (Macdonald et al. 2009, Wakelin et al. 2008). In recent years, soil physical and chemical properties have been measured and interpreted in light of soil biochemistry and microbial communities in studies across a range of disturbance scenarios (e.g. Romaniuk et al. 2011, Lopes et al. 2011, Cookson et al. 2008a, Wakelin et al. 2008). Most of these studies focused on detecting differences among experimental treatments and determining which methods were most useful for this rather than on relationships among soil characteristics. A sound understanding of the associations among soil chemical properties, and catabolic potential and enzymatic activity of soil would facilitate construction of an indicator or indicators of the functioning of microbial communities. This would be particularly useful in the context of both management of remaining remnant woodlands in *E. globulus* plantations and revegetation of degraded remnant woodlands.

One of the possible candidates for such an indicator may be an index of functional diversity. Functional evenness, which indicates the utilization of the resources in a niche and thus productivity (Schleuter et al. 2010) has been used with some success (Romaniuk et al. 2011, Lalor et al. 2007). The Shannon-Weaver index has been used widely as a measure of diversity in plant and animal communities as it combines a range of variables into a single value (Izsak and Papp 2000). A similar approach using functional diversity of soil
biochemical properties has been tested recently (Marinari et al. 2013). In their study, the Shannon-Weaver diversity index (SWI) was calculated using soil enzymatic activity data to successfully detect differences among treatments. However, Lopes et al. (2011) did not find differences between conventional and organic farm management treatments using the SWI calculated from catabolic potential profiles and bacterial community PCR-DGGE fingerprints. The usefulness of this novel approach in the context of Australian soils and vegetation is yet to be tested.

The experiments described in Chapters 3 to 5 and in Appendix I have provided data from a range of treatments probing both degradation processes and restoration practices associated with land use change. A reference data set for five land use conditions spanning intact native woodland through to native vegetation remnants to pasture and E. globulus plantations has also been assembled. Although effects of land condition and restoration and degradation treatments on soil properties were found, the data sets generated were analysed in isolation and interpretation of the patterns found were made in the context of changes in vegetation for a specific process or practice. The scope of the current study was not wide enough to develop and investigate soil indicators; however, a preliminary analysis of combined datasets, that could reveal soil properties with potential use in future work on a soil indicator, was not unjustified. For such a generalised analysis, soil chemical and biochemical properties were reanalysed to achieve three objectives. The first was to investigate the usefulness of a functional diversity approach to assess the effects of treatments in the restoration and degradation trials (Chapter 4 and 5 and Appendix I). The hypothesis to be tested was that the SWI can be used to distinguish among the treatments applied and land conditions used.

The second was to determine if patterns of change in soil properties found to occur during manipulative trials (Chapter 4 and 5 and Appendix I) are related to soil characteristics of the five reference land conditions (Chapter 3), and to detect soil properties most indicative
of land condition and changes due to degradation and restoration treatments. The hypothesis was that a small number of biochemical soil properties change in response to degradation processes and restoration treatments and may therefore be useful in development of a potential indicator of land condition and change. Multiple factor analysis (MFA) was used to investigate latent structures in the data, and soil properties representative of them.

The third objective was to reveal those associations between soil properties that could be identified with, and subsequently serve as potential indicators of, soil nutrient pools and nutrient cycling. It can be hypothesised that soil catabolic potential will correlate with soil enzymatic activity and chemical properties in patterns representative of cycling of C, N, P and S and can be used as a substitute for enzymatic activity assays, which would streamline laboratory analysis. Variation partitioning and redundancy analysis (RDA) was used to investigate the relationships among soil properties. Additionally, this approach could be used to determine which soil chemical properties influence soil biochemical properties.

6.2. **Materials and methods**

6.2.1. **Data collection and pre-treatment**

The analysis was done on a subset of soil properties that were tested in all experiments described in Chapters 3 to 5 and Appendix I (Table 6.1). These variables (n = 24) were used to construct the following five data sets (see Fig. 6.1):

1. five reference land conditions data (Chapter 3),
2. end-point data of the degradation experiment (Chapter 4),
3. end-point data of the pilot restoration study (Appendix I)
4. end-point data of the restoration study (Chapter 5), and
5. combined data sets from (1) to (4).

Detailed description of the data collection is given in the relevant chapters.
Table 6.1. Soil properties (abbreviated in parentheses) used in the analyses in Chapter 6.

<table>
<thead>
<tr>
<th>Enzymatic activity</th>
<th>N-mineralization potential</th>
<th>Catabolic potential substrates</th>
<th>Chemical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arylsulphatase (AR)</td>
<td>Ammonium generated in 7 days of incubation (NminNH4)</td>
<td>water (w)</td>
<td>Moisture (M)</td>
</tr>
<tr>
<td>Urease (UR)</td>
<td></td>
<td>succinic acid (sa)</td>
<td>Ammonium (A)</td>
</tr>
<tr>
<td>Deaminase (DE)</td>
<td></td>
<td>thiamine (t)</td>
<td>Nitrate –N (NitN)</td>
</tr>
<tr>
<td>Alkaline phosphomonoesterase (AlP)</td>
<td></td>
<td>cinnamic acid (c)</td>
<td>Potassium (K)</td>
</tr>
<tr>
<td>β-glucosidase (BG)</td>
<td></td>
<td>α-ketoglutaric acid (k)</td>
<td>Conductivity (Cond)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D-galacturonic acid (g)</td>
<td>Phosphorus (P)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>imidazole (i)</td>
<td>Organic C (Orgc)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sulphur (S)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pH in CaCl₂ (pH)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total N (TotN)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ammonium by microplate method (Amp)</td>
</tr>
</tbody>
</table>

6.2.2. Statistical analyses

The variables were transformed to improve normality of distributions. Nominal factors used in analyses were: site and a combined “treatment” factor, consisting of land condition from data set 1 and experimental treatments from data sets 2 to 4.

Figure 6.1. Schematic diagram of the construction of data sets analysed in Chapter 6.
Functional diversity

Variables in data sets 1 to 4 were ranged within each data set according to Marinari et al. (2012) to eliminate bias arising from differences in the scale of measurements of different soil properties and different timing of experiments. Subsequently, the Shannon-Weaver index (SWI) was calculated from all variables for each sample. The effects of treatment and site on the SWI values were investigated using analysis of variance (ANOVA) with fixed factors and Tukey’s Honestly Significant Difference tests for multiple comparisons. The Levene’s test was used to test that the homoscedasticity assumption for ANOVA was met. The models included two factors, in one-way design for data sets 1 and 3 and completely crossed design for data sets 2 and 4. The fit of the models was verified by inspection of the plots of residuals against fitted values.

Multivariate analyses

A multiple factor analysis (MFA) was done using data set 5 as described in Chapter 3. For clarity, a detailed description is only given for the dimensions clearly discerning between treatments. Site, experiment and treatment were included in the analysis as nominal factors.

The grouping of variables in data set 5 was investigated using redundancy analysis with the matrix of correlations between the variables calculated using Kendall’s Rank Method (Quinn and Keough 2003).

The variability in biochemical soil properties that could be attributed to the variability in chemical soil properties was investigated using redundancy analysis for enzymatic activity and catabolic potential tested separately and in combination. Data were standardised to meet the assumptions of the procedure for automatic forward selection of explanatory variables (Borcard et al. 2011). Data set 5 was split into three subsets of variables, comprised of enzymatic activity with N-mineralization potential, catabolic potential, and chemical properties with moisture. The significance of the full models was tested using a
permutational test with 999 permutations, and when significant, parsimonious models were constructed from the full models using an automatic forward selection procedure, as described in Borcard et al. (2011). Tests for significance of the models were obtained during forward selection using 199 permutations. The most parsimonious models were investigated in detail using variation partitioning and RDA.

The statistical analysis and preparation of the graphic output was performed in R using packages vegan (Oksanen et al. 2011) and FactoMineR (Husson et al. 2011).

6.3. Results

6.3.1. Functional diversity in soil from contrasting land conditions and treatments

The differences in the Shannon-Weaver Index due to treatment factor were detected by ANOVA only in data set 1, i.e. in reference land condition data ($F_{4,12} = 7.19$, $MS_{resid} = 0.008$, $p = 0.003$). For all other data sets, the results were not significant (data not shown). The SWI for soil from _E. globulus_ plantations was lower than soil from all other sites except intact native woodland (Fig. 6.2); however, the Shannon-Weaver index values in the former group were highly variable, which masked the differences between other land conditions and caused violation of the assumption of the homoscedasticity ($Levene’s$ test statistic = 3.2, $p = 0.043$). When the Shannon-Weaver index data for _E. globulus_ plantation were omitted from the analysis, the assumptions of the ANOVA were met ($Levene’s$ test statistic = 1.78, $p = 0.203$), and the differences in the Shannon-Weaver index values between the remaining land conditions were significant ($F_{3,9} = 10.2$, $MS_{resid} = 0.003$, $p = 0.003$). The soil from intact native woodland had significantly lower Shannon-Weaver index than soil from the woodland remnants and pasture (data not shown).
**Figure 6.2.** Differences in functional diversity based on the Shannon-Weaver index calculated from soil chemical and biochemical properties among five land conditions in data set 1. Same letter denotes no statistical difference at the level of $\alpha = 0.05$; error bars represent standard deviation of the mean ($n = 4$). IN woodland – intact native woodland; UDN – remnant with native understory; UDE – remnant with exotic understory.

### 6.3.2. Comparisons among experimental treatments in reference to land condition

The first 29 dimensions of MFA were significant according to the average eigenvalue model (data not shown), and cumulatively explained 93% of the total variability. Of these, only dimensions 7, 8, 10 and 12 revealed clear separation between treatments in all data sets and are reported in detail. Each of these dimensions alone explained only slightly more than 2.5% of the total variability.

Dimensions 7 and 8 separated land conditions dominated by exotic *versus* native vegetation in data set 1 (Fig. 6.3 A), fertilized *versus* non-fertilized plots in data set 2 (Fig. 6.3 B), and selected restoration treatments in data set 3 (Fig. 6.3 C). Restoration treatments in data set 4 were separated only along Dimension 7 (Fig. 6.3 D). Dimension 10 separated pasture from intact native woodland and *E. globulus* plantations, with remnants in between these two land conditions in data set 1 (Fig. 6.4 A); non-cleared *versus* cleared *versus*
fertilized soils in data set 2 (Fig. 6.4 B); selected restoration treatments in data set 3 (Fig. 6.4 C) and soil from control versus treated plots in data set 4 (Fig. 6.4 D).

Dimension 12 separated *E. globulus* plantations, remnants with exotic understorey and the three remaining land conditions in data set 1 (Fig. 6.4 A), and the restoration treatments in data sets 3 and 4 (Fig. 6.4 C and D). Differences among sites were detected along the reported dimensions and were particularly clear in data sets 2 and 4 (Fig. 6.3 and 6.4 B and D).

Additionally, the results of the degradation and restoration trials (data sets 2-4, Fig. 6.3 B-D and 6.4 B-D) were compared to the reference data set (data set 1, Fig. 6.3 A and 6.4 A) to determine which treatments caused shifts in remnant’s soil characteristics from exotic to native understorey (henceforth “UDE to UDN”) or native to exotic understorey (henceforth “UDN to UDE”). These changes could be interpreted as improvement or degradation of the condition of the remnant woodland, respectively.

For data set 2, fertilizer addition caused UDN to UDE along Dimensions 7 and 8, particularly after taking into account the effect of site. Clearing caused UDN to UDE along Dimension 8. An opposite trend was found in Dimension 10. In data set 3, the following treatments: glyphosate, simazine with glyphosate and planting, and glyphosate with harvest residue mulch and planting resulted in UDE to UDN along Dimension 7, but along Dimension 8 most treatments caused UDN to UDE. A similar trend was detected in data set 4 for both black plastic and harvest residue mulch treatments. Moreover, in the same data set, the planted treatments: glyphosate with simazine and glyphosate with harvest residue mulch caused UDE to UDN along Dimension 10. Along Dimension 12, the former treatment led to UDE to UDN, while glyphosate with harvest residue mulch led to UDN to UDE. For data set 4, black plastic, harvest residue mulch and glyphosate treatments resulted in UDE to UDN along Dimension 10; however, along Dimension 12 harvest residue mulch and black plastic had the same effect.
Figure 6.3. Differences in soil biochemical properties among treatments and land conditions detected for MFA Dimensions 7 and 8. (A) data set 1: IN – intact native woodland; UDN – native woodland remnant with native understorey; UDE – native woodland remnant with exotic understorey; (B) data set 2: colour code for site: black – Mi; red – R; green – RG; (C) data set 3: C – non-treated control; R – glyphosate; S – mulch from E. globulus harvest residue; SimR – simazine and glyphosate; SR – glyphosate and mulch from E. globulus harvest residue; +p – with plantings; (D) data set 4: colour code for sites: dark blue – Ma; light blue – P; purple – S. Dashed lines represent distances from the group centroids; continuous ellipses represent the values of one standard deviation from the group centroids; the percentage of the variability in the MFA model explained by the dimensions is given in parentheses.
Figure 6.4. Differences in soil biochemical properties among treatments and land conditions detected for MFA Dimensions 10 and 12. (A) data set 1: IN – intact native woodland; UDN – native woodland remnant with native understorey; UDE – native woodland remnant with exotic understorey; (B) data set 2: colour code for site: black – Mi; red – R; green – RG; (C) data set 3: C – non-treated control; R – glyphosate; S – mulch from *E. globulus* harvest residue; SimR – simazine and glyphosate; SR – glyphosate and mulch from *E. globulus* harvest residue; +p – with plantings; (D) data set 4; colour code for sites: dark blue – Ma; light blue – P; purple – S. Dashed lines represent distances from the group centroids; continuous ellipses represent the values of one standard deviation from the group centroids; the percentage of the variability in the MFA model explained by the dimensions is given in parentheses.
Dimensions 7 and 8 were represented by similar soil properties as Dimensions 10 and 12, namely: total soil N, ammonium (detected using conventional assays and microplate method), N-mineralization potential, respiration in response to succinic and cinnamic acid and K and S concentrations. The correlations of these variables to the dimensions were significant at the level of $\alpha = 0.05$ (Fig. 6.5). Organic C, pH and P concentration represented Dimension 7 in addition to the variables listed above (Fig. 6.5).

### 6.3.3. Relationships among soil properties

The first three canonical axes (RDAs), resulting from the redundancy analysis of the matrix of correlations between the variables, explained nearly 74% of the variability in data set 5 and revealed several groupings of soil properties (Fig. 6.6). N-related variables ($\text{TotN, NitN, UR and Amp}$), as well as P concentration and $\beta$-glucosidase activity were separated from the catabolic potential variables along RDA1, while alkaline phosphomonoesterase activity and respiration in response to water were separated from the remaining variables along RDA2 (Fig. 6.6). Catabolic potential variables were separated from $\text{OrgC, TotN, deaminase activity and conductivity along RDA3}$ (Fig. 6.6). This ordination placed arylsulphatase and deaminase activity, and soil S and inorganic N concentration on opposite ends of RDA1. A similar pattern was found for alkaline phosphomonoesterase and soil P. Soil pH was located away from the other soil properties (Fig. 6.6).

A significant proportion of the variability in soil biochemical properties was explained by soil chemical properties: $F_{11,129}= 12.6, 5.11$ and 7.9 for the enzymatic data set, catabolic potential data set and both data sets combined, respectively; $p <0.001$ in all three cases. Forward selection of explanatory variables resulted in similar subsets of predictor variables (Table 6.2), which explained 21%, 12% and 16% of total variability in the enzymatic, catabolic potential and all data sets combined, respectively (Table 6.3).
Figure 6.5. Correlation among soil properties and dimensions resulting from MFA analysis of data set 5; (A) Dimensions 7 and 8; (B) Dimensions 10 and 12 that were significant at the level of $\alpha = 0.05$. Abbreviations: AlP- alkaline phosphomonoesterase activity, P- phosphorus, K- potassium, S- sulphur, OrgC-organic carbon, TotN- total nitrogen, pH- pH in $\text{CaCl}_2$, Amp- ammonium by microplate method, A- ammonium-N, NminNH4- N-mineralization potential; respiration in response to: sa- succinic acid, i- imidazole, t- thiamine, c- cinnamic acid.
Figure 6.6. Grouping of soil biochemical properties detected by redundancy analysis in data set 5. The percentage of total variability in the data set explained by the canonical axes is given in parentheses. Abbreviations: Amp- ammonium by microplate method, M-moisture, A-ammonium-N, NitN- nitrate-N, P- phosphorus, K- potassium, S- sulphur, OrgC- organic carbon, TotN- total nitrogen, Cond- conductivity, pH- pH in CaCl₂, NminNH₄- N-mineralization potential; activity of: AR- arylsulphatase, UR- urease, DE- deaminase, AIP- alkaline phosphomonoesterase, BG- β-glucosidase; respiration in response to: ga- D-galacturonic acid, sa- succinic acid, k- α-ketoglutaric acid, i- imidazole, t- thiamine, c- cinnamic acid, w- water.

Ordination of the data from models obtained in the forward selection procedure revealed associations among chemical and biochemical soil properties (Fig. 6.7). The activity of β-glucosidase (BG), urease (UR) and arylsulphatase (AR) was related to soil N, P and OrgC concentration, while that of deaminase (DE) was related to soil conductivity (Fig. 6.7 A). For the catabolic potential data, respiration in response to imidazole was related to soil inorganic N (NitN and Amp), while respiration in response to organic acids was related to ammonium (A) and pH (Fig. 6.7 B). The ordination of the combined data revealed that soil N, P and C were related to enzymatic activity along the
first canonical axis, and conductivity, pH and DE activity were related to soil catabolic potential along the second canonical axis (Fig. 6.7 C). Such grouping of the variables measured generally resembled the RDA of the matrix of correlations when all variables were compared (Fig. 6.6).

Table 6.2. Forward selection of variables explaining variability in the biochemical soil properties in data set 5. In all cases, the degrees of freedom = 1.

<table>
<thead>
<tr>
<th>Response</th>
<th>Soil chemical property</th>
<th>Adjusted R²</th>
<th>AIC</th>
<th>F</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil enzymatic</td>
<td>+ NitN</td>
<td>0.17</td>
<td>229.09</td>
<td>28.80</td>
<td>0.005</td>
</tr>
<tr>
<td>properties</td>
<td>+ TotN</td>
<td>0.26</td>
<td>214.00</td>
<td>17.78</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>+ pH</td>
<td>0.31</td>
<td>203.56</td>
<td>12.63</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>+ OrgC</td>
<td>0.36</td>
<td>193.98</td>
<td>11.65</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>+ M</td>
<td>0.40</td>
<td>186.45</td>
<td>9.44</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>+ Amp</td>
<td>0.44</td>
<td>178.43</td>
<td>9.87</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>+ Cond</td>
<td>0.47</td>
<td>171.97</td>
<td>8.23</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>+ P</td>
<td>0.47</td>
<td>170.49</td>
<td>3.30</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>All variables</td>
<td>0.48</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Soil catabolic</td>
<td>+ pH</td>
<td>0.06</td>
<td>267.98</td>
<td>9.57</td>
<td>0.005</td>
</tr>
<tr>
<td>potential</td>
<td>+ NitN</td>
<td>0.12</td>
<td>258.62</td>
<td>11.57</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>+ Amp</td>
<td>0.15</td>
<td>255.57</td>
<td>5.00</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>+ M</td>
<td>0.18</td>
<td>252.05</td>
<td>5.43</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>+ A</td>
<td>0.19</td>
<td>250.81</td>
<td>3.14</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>+ K</td>
<td>0.20</td>
<td>250.13</td>
<td>2.57</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>+ P</td>
<td>0.21</td>
<td>248.64</td>
<td>3.33</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>All variables</td>
<td>0.24</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>All soil</td>
<td>+ NitN</td>
<td>0.10</td>
<td>349.05</td>
<td>16.27</td>
<td>0.005</td>
</tr>
<tr>
<td>biochemical</td>
<td>+ pH</td>
<td>0.16</td>
<td>339.47</td>
<td>11.81</td>
<td>0.005</td>
</tr>
<tr>
<td>properties</td>
<td>+ M</td>
<td>0.22</td>
<td>330.79</td>
<td>10.78</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>+ Amp</td>
<td>0.25</td>
<td>325.45</td>
<td>7.26</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>+ TotN</td>
<td>0.28</td>
<td>320.37</td>
<td>6.95</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>+ OrgC</td>
<td>0.31</td>
<td>316.07</td>
<td>6.13</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>+ Cond</td>
<td>0.33</td>
<td>313.48</td>
<td>4.40</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>+ P</td>
<td>0.34</td>
<td>311.87</td>
<td>3.42</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>+ K</td>
<td>0.35</td>
<td>311.53</td>
<td>2.19</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>All variables</td>
<td>0.35</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
</tbody>
</table>

Table 6.3. Variation in the soil biochemical properties explained by soil chemical properties as detected by variation partitioning in data set 5.

<table>
<thead>
<tr>
<th>Soil properties</th>
<th>Factor</th>
<th>Adjusted R²</th>
<th>F</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic activity</td>
<td>Chemical properties + Site</td>
<td>0.56</td>
<td>12.36</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Chemical properties</td>
<td>0.21</td>
<td>9.06</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Site</td>
<td>0.09</td>
<td>4.41</td>
<td>0.001</td>
</tr>
<tr>
<td>Catabolic potential</td>
<td>Chemical properties + Site</td>
<td>0.32</td>
<td>5.47</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Chemical properties</td>
<td>0.12</td>
<td>4.25</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Site</td>
<td>0.11</td>
<td>3.76</td>
<td>0.001</td>
</tr>
<tr>
<td>All biochemical properties</td>
<td>Chemical properties + Site</td>
<td>0.43</td>
<td>7.30</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Chemical properties</td>
<td>0.16</td>
<td>5.12</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Site</td>
<td>0.09</td>
<td>3.54</td>
<td>0.001</td>
</tr>
</tbody>
</table>

a – nitrate N, total nitrogen, pH in CaCl₂, organic carbon, moisture, ammonium by microplate method, conductivity, phosphorus; b – pH in CaCl₂, ammonium by microplate method, nitrate N, moisture, ammonium-N, potassium, phosphorus; c – nitrate N, pH in CaCl₂, moisture, ammonium by microplate method, total nitrogen organic carbon, conductivity, phosphorus, potassium.

6.4. Discussion

6.4.1. Functional diversity

In the current study, the Shannon-Weaver index was useful for distinguishing land use condition but had limited use when restoration or degradation processes were examined. Soil properties selected in this experiment were suitable to detect differences between land conditions, but not between experimental treatments. When considering the usefulness of the SWI in assessing soil disturbance, these results fall in-between the outcomes of the studies of Marinari et al. (2012), which advocated this approach, and of Lopes et al. (2011), which found no support for its use. A possible explanation for these differences may be that the reference data set encompassed soils with very different characteristics as they were from different land conditions, while the soils in the manipulative experiments were much more similar to each other as they involved a single land condition. Moreover, when the Shannon-Weaver index was calculated using all of the biochemical properties assayed, even the differences among treatments in the references data set were no longer apparent (data not shown). In addition, there were no differences according to treatment when the Shannon-Weaver index was calculated separately for the three subsets of variables used in the multiple factor analysis. This
indicates that the choice of variables used for calculation of diversity is highly influential. The use of the Shannon-Weaver diversity index calculated from the soil properties tested here is therefore not ideal for monitoring and assessment of remnant health and restoration processes.

For soil biochemical properties, including enzymatic activity and catabolic potential, the Shannon-Weaver index was higher in areas with a mixture of native and exotic plant species, as compared to areas without exotic species. Marinari et al. (2013) reported that younger, undeveloped soil, with a variety of soil substrates and chemical and biochemical processes found in the profile, and higher entropy, had higher "enzymatic" diversity than mature soils. In light of this argument, the current results imply that in the reference data set, the presence of exotic plant species was concurrent with increased entropy and microbial functional diversity. This could be a result of exotic plants mobilising soil resources untapped by the native organisms and thus increasing the range of substrates available for microbial utilisation. However, the current studies could not ascertain that the reason for such an increase was the suggested enrichment of soil biochemistry due to the introduction of the exotic microbial populations, associated with exotic plant species; as opposed to the reverse, i.e. the changes in soil chemistry creating favourable conditions for the invasion of exotic plant species. This alternative explanation is plausible especially in consideration of the results from the experimental degradation and restoration treatments, where the Shannon-Weaver index did not change.
Figure 6.7. Relationships between soil chemical and biochemical properties detected by redundancy analysis. Weighted average scores are given for (A) enzymatic activity; (B) catabolic potential and (C) enzymatic activity and catabolic potential combined. Continuous lines represent response variables; dashed lines represent explanatory variables. The percentage of the variability explained by the canonical axes in the RDA models is given in parentheses. Abbreviations: AlP- alkaline phosphomonoesterase activity, P- phosphorus, K- potassium, S- sulphur, OrgC- organic carbon, TotN- total nitrogen, pH- pH in CaCl₂, Amp- ammonium by microplate method, A- ammonium-N, NminNH4- N-mineralization potential; respiration in response to: sa- succinic acid, i- imidazole, t- thiamine, c- cinnamic acid.
6.4.2. Differences in soil properties between experimental treatments in the context of reference land conditions

The investigation of latent structures in the data sets using multiple factor analysis revealed that land condition was not a major source of variability. This suggests that there may be other factors that have greater influence on soil biochemical properties, such as, for example, soil physical properties (Chapter 3). However, a good separation of treatment groups was detected in case of several MFA dimensions. Soil ammonium concentration, along with K and S concentration, N-mineralization potential and respiration in response to imidazole, succinic acid and cinnamic acid, varied among land condition and experimental treatments, which suggests their potential usefulness in assessing changes in soil biochemistry. Surprisingly, soil pH and organic C were poor indicators of change, given their lack of correlation with multiple factor analysis dimensions in this study. The only enzymatic activity associated with multiple factor analysis dimensions was that of alkaline phosphomonoesterase.

The subset of best predictors of treatments effect in multiple factor analysis was not the same as those identified by redundancy analysis. This implies that the treatments used in the degradation and restoration studies did not affect the main soil properties responsible for the changes in biochemical properties. For example, soil nitrate concentration was not well correlated to dimensions in multiple factor analysis analyses even though it was one of the most important predictors of variability in the soil biochemical properties in redundancy analysis. The experimental treatments used in this study did not introduce marked variability in this measure, and only a small proportion of the total variability in the data was explained by the effects of the experimental treatments, implying that soil nitrate was not a useful measure for monitoring changes occurring in soil subsequent to these restoration treatments. In the study by Macdonald et al. (2009), soil nitrate had a strong influence on soil microbial communities, as opposed to soil ammonium. This would suggest that unless soil nitrate content is altered,
other soil biochemical properties that rely on microbial activity are unlikely to change, and that an effective restoration treatment would be the one that alters the main drivers of microbial functioning, such as soil nitrate content. The current study shows the importance of measuring a range of soil and biochemical properties and understanding their usefulness in differentiating among land use type and restoration success. Considering that the magnitude of differences in soil properties resulting from the application of various restoration techniques was smaller than that due to land conditions, a single set of analyses for answering two very different questions, however desirable, was not feasible at this point.

In addition, multiple factor analysis showed that soils subjected to the experimental treatments and sourced from different land conditions differed only in a handful of soil properties. This suggested that it is possible to arrive at a minimal data set of indicators for the assessment of remnant health or performance of restoration and degradation treatments using the laboratory and statistical methods described in the current work.

The usefulness of the current analyses for assessing the efficacy of the restoration and degradation treatments could be inferred from the similarities and differences of the characteristics of the soils under experimental treatments to the reference soils. The results of these comparisons were not consistent: the position of the control samples in the ordination plots and shifts observed did not always agree with the simplistic assumption of the linear differences between the land use conditions. An explanation for this fact may be that MFA in essence resulted in detailed investigation of only a few soil properties, that were most variable when the constraint of the treatment was imposed on the data, so the resulting ordination may not be detailed enough to discern sufficiently between all treatments and is thus unclear. It is possible that with a different set of soil properties a more unambiguous result could be obtained.
6.4.3. Relationships between chemical and biochemical soil properties

RDA analyses revealed two tight clusters of variables: most of the enzymatic activity measures with soil N and P content, and catabolic potential measures with soil K and S content (Fig. 6.2 and 6.3). This implies that the enzymes selected for analysis were a good representation of changes in inorganic N and P in soil, possibly as a result of involvement in cycling of these nutrients. Similarly, catabolic potential measures were well characterised by S and K concentrations in soil. It is evident that neither enzymatic activity nor catabolic potential can be reliably used as indicators to predict changes in total N, ammonium or organic C in soil in the land use types tested. This is in contrast with findings from Cookson et al. (2008b) where catabolic potential profiles in rehabilitated forest soils in Western Australia were strongly dependent on soil organic C and ammonium concentration.

Investigation of the redundancy analysis ordination for alkaline phosphomonoesterase activity and soil P concentration, or deaminase activity and soil ammonium concentration suggested that the product of the enzymatic activity did not inhibit the enzyme. Arylsulphatase activity was more closely related to P and nitrate concentrations in soil than to S, suggesting an interdependence of the S, N and P cycling. A similarly close association was found between deaminase activity and soil organic C, which was expected, as soil organic matter is a likely source of organic N serving as the substrate for this enzyme (Killham and Rashid 1986).

The potential for substituting catabolic potential tests for enzymatic assays and vice versa to streamline analytical protocols and reduce the number of analyses required was not possible. This is not surprising as enzymatic activity assays and catabolic potential tests measure dissimilar and largely incomparable processes. The set of catabolic potential measures investigated in the experiments described herein was more related in redundancy analysis to soil S and K content than was the set of enzymatic activities, while the opposite was found for soil N and P content. In conjunction with the
multiple factor analysis results, which implied that S and K, but not N and P, were useful for discerning between experimental treatment groups, redundancy analysis suggests that the catabolic potential is more useful than enzymatic activity for detection and assessment of the effects of restoration treatments. This is in agreement with the work of Cookson et al. (2008a) and Lalor et al. (2007), who successfully used the catabolic potential assays to discern between the effects of restoration treatments on soils from jarrah forest rehabilitated after mining in WA.

There were several findings in this study that were particularly interesting when compared with results of other Australian studies. For example, pH was one of the major explanatory factors for the variability in catabolic potential data. This soil property was found to be the main driver of microbial diversity and changes in catabolic potential for a range of Australian soils (Wakelin et al. 2008) and in microbial PLFA in UK soils (Rousk et al. 2010). On the other hand, Cookson et al. (2008a) determined that soil organic C, ammonium and nitrate were the main factors influencing catabolic potential profiles in soil from forests in Western Australia. In the current study, soil nitrate was a more influential explanatory factor for the variability in the enzymatic activity data, and all soil properties tested together, than ammonium. Macdonald et al. (2009) found that microbial communities in pasture soils in Western Australia were influenced by soil nitrate, but not by ammonium. Also, in the current study, soil moisture had a significant impact on microbial activity, similarly to what was found in studies by Wakelin et al. (2008) and Macdonald et al. (2009). Such beginnings of convergence of functional and molecular results for different types of Australian soils suggest the possibility of global patterns of responses by the microbial community to disturbances in their environment.

6.5. Conclusions

The analyses described here provide the first account of relationships between soil chemical and biochemical properties in relation to restoration and degradation
processes in native vegetation remnants associated with *E. globulus* plantations in the SWWA. Partitioning of soil properties into distinct groups of soil processes such as nutrient cycling was not possible with the available data. However, the analyses revealed distinct patterns of changes in soil properties with experimental treatments mimicking degradation and restoration processes. Several soil chemical and biochemical properties showed promise as potential indicators of land use type and of degradation and restoration processes; however, the design and limited scale of the current study eliminated the possibility of development of such indicators. More thorough and larger scale studies would be necessary before benefits for the end-users would become clear. There are prospects that the measurement of physical and biochemical properties of soil is a useful tool for monitoring the condition of the native vegetation remnants and the efficiency of the restoration treatments, but the specific variables tested and analyses used will differ according to management needs and questions.
Chapter 7. Synthesis

7.1. Major findings of the study

The study presented here investigated the usefulness of a range of soil physical, chemical and biochemical properties as firstly, a means of determining the differences among a range of land use conditions common in SWWA, secondly, assessing the effects of degradation processes affecting the native vegetation remnants within *E. globulus* plantations, and thirdly, investigating the efficacy of relevant restoration practices (Fig. 7.1). The main findings from this study were:

a) A range of high throughput microplate tests, which are less time-consuming and less costly than the conventional, larger-scale approaches to the analysis of biochemical soil properties, was useful in detecting differences among soils from different land uses and experimental treatments (Table 7.1). These results confirmed the utility of the microplate tests approach in restoration ecology.

b) Differences among land condition and changes due to the experimental treatments simulating degradation and restoration of native vegetation remnants could be detected by a small number of soil chemical and biochemical properties (Table 7.1).

c) The main sources of variability in soil biochemical properties were soil physical properties (Chapter 3) and soil pH, nitrogen content (nitrate in particular) and moisture (Chapter 6).

d) Nutrient enrichment in the form of fertilizer addition was more influential on soil biochemical properties than other degradation-related agents, such as changes in vegetation cover (Chapter 4).

e) There were several species-specific responses by native vegetation to the treatments tested for remnant restoration (Chapter 5, Appendix I).
f) The use of mulch with high C content and replanting with seedlings of native understorey plants in canopy openings are recommended methods for restoration of remnants of native vegetation with an exotic understorey in *E. globulus* plantations in SWWA.

Soil from remnants of native vegetation in different condition enclosed within *E. globulus* plantations can be distinguished by chemical and biochemical tests in microplate format supporting Hypothesis 1 (Chapter 1.5). Confirmation of the usefulness of the microplate format for a range of assays provides support for it to become an inexpensive laboratory tool for assessment of potential restoration treatments and revegetation protocols, as well as for monitoring of restoration progress and land degradation processes. This has important implications for researchers in restoration ecology and soil microbiology and for managers in the plantation industry.

The impact of nutrient enrichment in native remnant vegetation in Australia cannot be underestimated with regards to degradation processes and restoration practices. In confirmation of Hypothesis 2, excess nutrients were found to be more influential on soil properties than clearing of native vegetation or temporary incursion of exotic plant species (Chapter 1.5). The continued presence of exotic plant species was not sustained for the duration on the experiment even in fertilized plots suggesting that the level of disturbance in nitrogen cycling caused in the current study was not enough to permanently alter soil status. This implies that to maintain the state of soil in native woodland remnant vegetation, excess nutrients, particularly N, must be avoided; otherwise an incursion of exotic plant species may become permanent.
Figure 7.1. Schematic representation of the study.
Table 7.1. Soil properties most useful in discernment between land conditions and experimental treatments in this study.

<table>
<thead>
<tr>
<th>Soil properties</th>
<th>Land condition (Chapter 3)</th>
<th>Degradation experiment (Chapter 4)</th>
<th>Restoration experiment (Chapter 5)</th>
<th>Combined data sets (Chapter 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrogenase (DHE)</td>
<td></td>
<td>Alkaline phosphomono-esterase (AIP)</td>
<td>Alkaline phosphomono-esterase (AIP)</td>
<td>Alkaline phosphomono-esterase (AIP)</td>
</tr>
<tr>
<td>Phenol oxidase (PO)</td>
<td>Arylsulphatase (AR)</td>
<td>Arylsulphatase (AR)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-glucosidase (BG)</td>
<td>β-glucosidase (BG)</td>
<td>β-glucosidase (BG)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urease (UR)</td>
<td>Deaminase (DE)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N-mineralization</td>
<td>Ammonium by microplate method</td>
<td>-</td>
<td>Ammonium by microplate method</td>
<td></td>
</tr>
<tr>
<td>Biochemical (microplate assays)</td>
<td>N-mineralization potential</td>
<td>Nitrate by microplate method</td>
<td>Nitrate by microplate method</td>
<td>N-mineralization potential</td>
</tr>
<tr>
<td>a-ketobutyric acid</td>
<td>cinnamic acid</td>
<td>D-galacturonic acid</td>
<td>cinnamic acid</td>
<td></td>
</tr>
<tr>
<td>D-galactose</td>
<td>α-ketoglutaric acid</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>DL-lactic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>imidazole</td>
<td>-</td>
<td>imidazole</td>
<td>imidazole</td>
<td></td>
</tr>
<tr>
<td>L-methionine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>L-rhamnose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Catabolic potential substrates</td>
<td>succinic acid</td>
<td>thiamine</td>
<td>thiamine</td>
<td>succinic acid</td>
</tr>
<tr>
<td>urocanic acid</td>
<td>-</td>
<td>-</td>
<td>thiamine</td>
<td>thiamine</td>
</tr>
<tr>
<td>Moisture* and chemical (external laboratory)</td>
<td>Moisture</td>
<td>Moisture</td>
<td>Ammonium-N</td>
<td></td>
</tr>
<tr>
<td>Nitrate-N</td>
<td>NitN</td>
<td>TotN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C:N ratio</td>
<td>-</td>
<td>Organic carbon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphorus</td>
<td>Phosphorus</td>
<td>Phosphorus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH in CaCl₂</td>
<td>pH in CaCl₂</td>
<td>pH in CaCl₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulphur</td>
<td>-</td>
<td>Sulphur</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* - in-house assay.

Not only did the biochemical properties of soil change in response to degradation-simulating treatments, they were also markedly altered by restoration treatments, supporting Hypothesis 3 (Chapter 1.5). Herbicide treatments and application of mulch altered soil properties and improved the success of native seedlings planted for
revegetation (Chapter 5, Appendix I). In addition, mulching with plantation harvest residue was found to be better than other treatments in terms of supporting survival and growth of planted native seedlings. In comparison to the herbicide treatment, mulch benefited a number of native plant species suggesting that this restoration practice is preferable over herbicide application.

In support of Hypothesis 4 (Chapter 1.5), a functional diversity index calculated from soil biochemical properties varied for soils collected from intact native woodland compared to soil from pasture and remnants of native vegetation. However, few differences in the functional diversity index were found for experimental treatments simulating degradation and rehabilitation processes. This suggested that scale and sensitivity is an important factor to consider when using soil functional diversity indices as measures of land use change. The recurrence of common soil properties in this study and other published studies, such as soil physical properties (Chapter 3) and soil pH and nitrate concentration (Chapter 6), as major determinants of variability in soil biochemical properties indicates the presence of global patterns, which warrants further studies of drivers of soil diversity in relation to land use change. Admittedly, the variability in soil biochemical properties due to soil type and physical properties, season and vegetation cover, and consequently, soil biological activity, makes an extrapolation of these results to other regions within Australia, and even within Western Australia, problematic. This variability can be at least partially overcome by conducting larger-scale, longer-term investigations, which are not available at present. Therefore, the results of the current study should be interpreted with caution.

7.2. Limitations of the study

7.2.1. Data collection

The time and cost of sampling and processing the samples using conventional methods, i.e. standard, traditional tests for soil chemical properties, were two major
constraints of this study, limiting the number of soil properties tested and the spatial scale and replication of the manipulative experiments. In addition, it was only possible to do experiments of short duration. The study sites were also located relatively close together and on soils of similar type; and the replication was minimal. These factors were unavoidable and are fully acknowledged in interpretation of the results. As a likely consequence, the effects of experimental treatments were found to be small. Nevertheless, this study was the first of this kind in WA. The results are applicable in a number of situations in the region, albeit a relatively small area. More broadly, the study has provided information about the variability of a range of soil properties, the sources of this variability, relationships among soil properties and the optimisation of laboratory methods.

7.2.2. Laboratory analyses

The choice of sample preparation method – whole soil or soil suspension – for the assays of soil biochemical properties, was important from the point of view of practicality and reproducibility of the results. Absorbance-based assays were used for determining soil enzymatic activity in the current study. These assays are much less sensitive and therefore require more concentrated samples than the fluorescence-based assays. Perhaps the biggest advantage of using absorbance-based, as opposed to fluorescence-based assays for enzymatic activity, is greater availability and low cost of substrates (Marx et al. 2001).

A high level of precision (CV less than 5%) of the microplate assays for nitrate and ammonium in soil extracts has been reported by Sims (1995) and Hood-Novotny (2010). In the current study, the precision of the assays for ammonium, measured on standard solutions in triplicate, was less than 7%. The variation measured is likely due to the volume of soil samples and reagents being two to three times smaller in the current study, which is likely to increase the risk of pipetting errors (Sims 1995). Estimations in extracts of triplicates from the same soil samples averaged the coefficient of variation to
18%. Since the precision of the assay was satisfactory (less than 10%), the high within-sample variability was attributed to inherent soil heterogeneity, as was the low precision, typically of around 20-30%, of the other assays for soil biochemical properties.

Despite their advantages, the reliability of the microplate assays for enzymatic activity is yet to be fully ascertained. For example, absorbance readings for soil samples were lower than ‘no-soil’ and ‘no-substrate’ blanks on several occasions. The no-soil blanks did not provide evidence of extensive product generation of non-biotic origin; however, the presence of the soil itself in the reaction mixture often resulted in higher absorbance than the amount of product generated by the enzymatic reaction. Evidence for this situation came from noticeable absorbance of no-substrate blanks. This is possible, considering the strength of extractants used in some of the assays (e.g. NaOH) used to stop the reaction in assays with pNP-linked substrates and the very heterogeneous nature of the soil (Franklin and Mills 2007). Assays for deaminase activity and soil respiration did not produce such artifacts. The assay for deaminase activity relied on substrate degradation rather than on product generation. For measurement of substrate-induced soil respiration, even though there were no negative “raw” results after the subtraction water-amended soil (i.e. the blank), some negative values were measured for some of the substrates used. This could be due to inhibition of soil respiration in response to these particular substrates. An in-depth investigation of this occurrence was beyond the scope of the current study and remains to be conducted.

Another issue related to the efficiency of sample processing to be addressed in the prospective experiments could be the use of multiwell plates with a filtering medium at the bottom of the wells for the assays in whole soil. In such an approach, products of the enzymatic reactions can be separated from the solids in the samples by filtration using vacuum or centrifugation, which further reduces sample handling and analysis time.
7.2.3. Interpretation of results

As in other studies (Macdonald et al. 2009, Cookson et al. 2008a, b, Wakelin et al. 2008), variability in chemical soil properties was used as a predictor of the variability in soil biochemical properties in this study. An inverse approach may also be legitimate. Changes in biochemical soil properties, such as enzymatic activity could potentially influence the pools of nutrients resulting from these enzymatic reactions. Similarly, the cause-effect relationships between the change in soil microbial activity and soil nitrate concentration, as found in the experimental treatments, could be bi-directional and are impossible to establish based on the results of the current study. To address these issues, long-term longitudinal and manipulative studies would be required. For example, an investigation of the impacts of the changes in soil enzymatic activity and catabolic potential on soil chemical properties through enrichment studies, for example using addition of catabolic potential substrates to soil, such as in Degens (1998), or of the activity of the community of ‘nitrifiers’ in the soil and their response to degradation and restoration treatments using functional genetic approaches.

7.3. Possible directions for future research

7.3.1. Biochemical indices of soil

The current study confirmed the usefulness of the CLPP approach in the restoration ecology context by showing the differences in catabolic potential among soil from different land conditions and some of the experimental treatments (Chapters 3 to 6). This was evident even though this particular approach was unable to identify substrates characteristic for all of the changes caused by the manipulative trials. Other studies pointed to maleic acid as a substrate indicative of changes due to restoration treatments in Western Australia (Cookson et al. 2008a, Banning et al. 2008), while a study in European soil reported response to α-ketoglutaric acid and glucose as characteristic to soils in disturbed and native condition (Romaniuk et al. 2011). Succinic and α-
Ketoglutaric acids were among the promising candidates in the current study. Potentially, testing other C and N sources and mixtures of them as substrates in the CLPP assays may result in finding more specific indicators for restoration processes for different soil types.

The data analyses presented in Chapter 6 highlighted that changes occurring in the soil due to the experimental treatments could be detected using a small number of chemical and biochemical properties of soil. Finding the minimum number of soil properties that are indicative of changes occurring during restoration and degradation, and possibly other processes such as those involved in soil contamination and remediation, thus seems to be possible. Such properties could be used to construct an index for monitoring degradation and restoration, maybe even using the functional diversity approach (Marinari et al. 2013). A first attempt of this was made in the current study, but the results were not as robust as desired. More intensively collected data from a broader range of land conditions using a suite of standardized protocols would likely prove to be successful in constructing such an index. Alternatively, the usefulness of other soil quality indices, based on biotic and abiotic soil characteristics and developed for different soils and climates, could be assessed (for review of possibilities see Sharma et al. 2011, Bastida et al. 2008, Schloter et al. 2003).

### 7.3.2. Investigation of restoration treatments

The current study found soil nitrate concentration to be one of the most important soil properties for differentiating between disturbed and undisturbed areas. However, in the degradation experiment (Chapter 4), soil nitrate levels did not change in the long-term and simulated degradation effects were not persistent. A decrease in soil nitrate was also associated with some of the restoration treatments tested. To further address this issue, an investigation of the ecology of nitrifying microorganisms in the soils and various methods that encourage denitrification is warranted. A useful technique would include application of a nitrification inhibitor (e.g. Andrews et al. 2011), and could lead to
development of efficient methods for reducing the weed load in native vegetation remnants.

Another way of limiting soil nutrient excess for rehabilitation purposes could be incorporation of charcoal as a soil amendment. Charcoal prepared from organic matter such as green waste or poultry litter, i.e. biochar, has been investigated intensively as a soil quality improver since the seminal paper by Lehmann et al. (2003). The majority of research to date has focused on the use of biochar as a source of nutrients for improved plant growth, as a modifier of soil physical properties and as a means of carbon sequestration. However, the literature also indicates that biochar can alter the availability of soil nutrients to plants, sometimes resulting in a decrease in crop growth (Lehmann et al. 2006, Lehmann and Rondon 2006). The investigation of the use of biochar as a method for restoration is also interesting from the practical perspective because biochar could be prepared, possibly even on-site, from plantation harvest residue. Such research would contribute to the understanding of the interactions among biochar and soil organic and inorganic matter, microorganisms and plants, which are still not fully understood (Joseph et al. 2010).

7.3.3. Alternative methodologies for rapid and inexpensive large-scale soil analysis

Investigations of soil genomics are an expanding area of study in soil ecology (Macdonald et al. 2009, Singh et al. 2009, Kasel et al. 2008, Wakelin et al. 2008). However, the associations among soil functioning, soil physical properties and the metagenome are still not fully understood. The current study focused on biochemical assays to assess microbial populations rather than on genetic molecular methods, as the latter are still too expensive to allow sampling on a routine basis and at a landscape-scale as would be required for monitoring of land use change. However, recent advances in DNA sequencing technologies (Schokralla et al. 2010, Schadt et al. 2010) have improved accessibility of the high-throughput sequencing of DNA, and statistical
tools developed for the analysis of the resulting high-volume genomic data, QIIME (Caporaso et al. 2010) and mothur (Schloss et al. 2009), are freely available.

Investigation of the molecular biology of the soils during the degradation and restoration experiments would provide important information about the diversity of the functional genes and about changes in microbial communities at the genomic level and would complement the biochemical analyses approach presented here.

The current study has demonstrated the usefulness of microplate assays for measuring soil biochemical properties when monitoring changes due to restoration and degradation. However, as efficient and cost-saving this approach may be, there is still a requirement for reagents and labour, which will be considerable if large, landscape-scale monitoring trials are attempted. The solution to this problem may be the analysis of near- and mid-infrared (NIR-MIR) absorbance/reflectance spectra, already used widely in soil science (e.g. Bellon-Maurel et al. 2010, Reeves 2010). Spectral analysis of soil can be used to detect differences among experimental treatments, but also to investigate the causes of these differences, such as changes in the activity of particular enzymes or alteration of nutrient concentrations. This approach has been used to investigate land condition (Cohen et al. 2006), disturbance of forest soil (Ludwig et al. 2002), the effects of heavy metal pollution on soil (Chodak et al. 2007) and correlations with CLPP profiles (Artz et al. 2006). Successful calibration of a range of soil properties of biological origin to the spectral data have been reported, including the activity of selected enzymes (Zornoza et al. 2008), the ratio of bacteria to fungi (Cecillion et al. 2009, Zornoza et al. 2008) and soil N-mineralization potential (Murphy et al. 2009, Russel et al. 2002). As an example of the broad range of application of this method, Vagen et al. (2006) developed a soil fertility index based on soil chemical properties, calibrated to both laboratory and Landsat reflectance data, which enabled mapping of the landscape-scale land use change. Even though NIR and MIR are predominantly used in this field of research, other methods of spectral analysis are available, such as Raman spectrometry, which in recent years has received increasing attention due to the technical developments that have
made it more reliable and readily available (Halvorson and Vikesland 2010). It has been used for non-invasive detection of chemicals in complex samples (Halvorson and Vikesland 2010, Ellis and Goodacre 2006), which could be of use in soil metabolomics research; and for identification of microorganisms, albeit so far for culturable microorganisms only (Harz et al. 2009, Maquelin et al. 2002).

The possibility of calibrating soil spectra with other data to predict soil properties based on the spectrophotometric measurements is particularly exciting. The potential lies in the ability to use high-throughput DNA sequencing in combination with soil chemical and biochemical properties for the appropriate calibrations. This suggests that with enough research and technological innovation, the use of conventional, laborious and time-consuming, wet chemistry-based assays for soil chemical and biochemical properties and molecular techniques for “–omic” analyses may ultimately be replaced by the faster, less laborious and cheaper method of spectrometry. This would facilitate data collection, the synthesis of landscape-scale functional and genomic information in soil, and the construction of widely applicable indices of soil status, which could be used in restoration ecology as well as in agriculture, forestry and other land management-related disciplines.

The majority of ecosystems bear the mark of human influence (Ellis et al. 2010, Ellis and Ramankutny 2008). Interfering with one part of complex interactions among organisms and their environment can have unpredicted consequences; hence there is an acute need for accurate tools for modelling, assessing and monitoring the possible outcomes of anthropogenic influences on ecosystems. The study presented here is a contribution towards addressing this by demonstrating that soil biochemical properties can be used as a tool to track land condition in south west Western Australia. This study provides a fundamental basis to enhance our understanding of the role of soil microbial functioning in land use change, degradation and restoration.
Bibliography:


Ecosystems and Human Well-being: Biodiversity Synthesis (2005) Millennium
Ecosystem Assessment. Washington, DC.: World Resources Institute. Forest
management—Economic, social, environmental and cultural criteria and
Australian Forestry Standard Limited.

Bonn, Germany: Forest Stewardship Council A.C.


Acosta-Martínez, V., & Tabatabai, M. A. (2000). Enzyme activities in a limed agricultural

microbial biomass of tallgrass prairie soil as related to burning and nitrogen
fertilization. Soil Biology & Biochemistry 31, 769-777.

Academic Press.

under the invasive nitrogen-fixing tree Falcataria moluccana. Soil Biology &

(2006). The ratio of fungi and bacteria in the biomass of different types of soil
determined by selective inhibition. Microbiology, 75(6), 702-707. doi:


Analytical Chemistry, 29(9), 1073-1081.


D. C. Coleman, D. F. Bezdicek & B. A. Stewart (Eds.), *Defining Soil Quality for a Sustainable Environment* (pp. 3-21).


Halvorson, R. A., & Vikesland, P. J. (2010). Surface-enhanced Raman spectroscopy (SERS) for environmental analyses. *Environmental science & technology, 44*(20),


Legendre, P., & Anderson, M. J. (1999). Distance-based redundancy analysis: Testing multispecies responses in multifactorial ecological experiments (vol 69, pg 1,


their management on soil food web properties and crop litter decomposition.


forest fragments of northeastern Brazil. *Biological Conservation, 141*(1), 249-260.


measure microbial biomass-C and biomass-N in soils from Western Australia.

*Soil Biology & Biochemistry, 25*(12), 1793-1801.


Appendix 1. Herbicides and harvest residue mulch in restoration of remnants with exotic understorey – pilot trial

This study investigated the effects of five restoration treatments on exotic ground cover, soil properties and survival and growth of seedlings of several native understorey species, following mulching with plantation harvest residue and/or spraying with herbicides.

8.1. Experimental treatments and data collection

The study sites were located between Mt. Barker and Albany in SWWA (see Chapter 2). Six native woodland remnants with exotic understorey were selected from four *E. globulus* plantation estates (sites) aged 7-10 years (C, P, L, Y, see Fig. 2.1, Chapter 2). The remnant represented by site P was different from the one chosen for the experiment described in Chapter 5. All sites had similar basic soil characteristics (Table 8.1). Within each remnant, 10 experimental plots (4 x 4 m) not encompassing living tree stems were established. Five restoration treatments were randomly assigned to the plots with two replicate plots per treatment: (1) non-treated control; (2) glyphosate: 1% v/v solution of Roundup™ (Monsanto Co., Columbus, OH) at 0.2 l m$^{-2}$, equivalent to 0.72 g m$^{-2}$ glyphosate; (3) mulching with *E. globulus* harvest residue; (4) glyphosate application as in (2) and subsequent mulching as in (3); (5) glyphosate as in (2) and simazine at 0.3 ml m$^{-2}$ (equivalent to 167 mg m$^{-2}$ simazine) with Pulse™ penetrant (Nufarm Australia Ltd) at 0.1% v/v (Table 8.2).

The treatments were applied in late May 2008, at the beginning of the wet season (winter). Two types of herbicides were applied using backpack sprayers: (1) glyphosate, a broad-spectrum herbicide with little or no effects on soil microorganisms in the field conditions (Weidenhamer and Callaway 2010, Busse *et al.* 2001); (2) simazine, an s-triazine herbicide which is widely used as a broad-spectrum pre-emergent herbicide and to control broadleaf weeds (Morgante *et al.* 2012). This compound is persistent in soil for
weeks to months depending on rainfall and soil type and has largely unknown effects on soil microbial communities. Coarse chip mulch (approximately 2 to 4 cm in diameter and <1 cm thickness) was obtained by processing *E. globulus* plantation harvest residue in a chipper. The mulch was spread evenly over the plots in a uniform layer of approximately 10 cm thickness. The plots in the combined mulch-glyphosate treatment were sprayed with glyphosate and mulched 2 weeks later. In July, 2 months after treatment application, one of the two replicate plots for each treatment within each site was planted with a mixture of native species seedlings commonly occurring in the remnants in the area. Seedlings of local native species: *Eucalyptus marginata* (ja), *Kennedia prostrata* (ke), *Hemiandria pungens* (he), *Agonis flexuosa* (ag), *Banksia grandis* (ba) and *Acacia pulchella* (ac) were obtained from a local nursery at 6 months of age. Eight seedlings of each species plus one randomly selected to total 49 seedlings per plot were planted in chosen plots, spaced regularly approximately 30 cm apart in a 7 x 7 grid. Planted plots were fenced to protect the seedlings from large herbivores.

Seedling survival (s), as a percentage of the initial number of seedlings planted, and average height (ah) was monitored for each species at 10 months after planting. Also, the following exotic ground cover (EC) measurements were taken independently by two observers: the percentage of ground covered by exotic species (tcov), exotic grasses (gcov) and by broadleaf exotic species (bcov), mostly *Phytolacca octandra* (inkweed), as well as the maximum (mht) and average (aht) height of the exotic plants within each plot. Topsoil was sampled to the depth of 5 cm using steel cores of 5 cm diameter from the plots before the commencement of the treatment application and at the end of dry season in late April the following year. Eight cores were collected from each plot, sealed in plastic bags and transported to the laboratory for the analyses of chemical and biochemical properties within 4 days after collection.
8.1.1. Laboratory analyses

Before treatment application, soil N-mineralization potential (NminNH4) and ammonium content tested in microplate format (Amp) were determined as described in Chapter 2.2.4 and the FDA hydrolytic activity assay (FH) was measured according to the Protocol 1 (Chapter 2.2.6). Soil chemical properties were determined as described in Chapter 2.2.3 before treatment application and at the end of the study. The following soil properties were determined at the end of the study: the activity of soil alkaline phosphomonoesterase (AlP), urease (UR), arylsulphatase (AR), deaminase (DE), β-glucosidase (BG), phosphodiesterase (PD), FDA hydrolytic activity (FH) and phenol oxidase (PO) was tested according to the Protocol 1 (Chapter 2.2.6). Soil respiration induced by water (w) and substrates: D-glucose (g), D-galacturonic acid (ga), succinic acid (sa), α-ketoglutaric acid (k), imidazole (i), thiamine (t) and cinnamic acid (c) was assayed as described in Chapter 2.2.5. Soil nitrogen mineralization potential assay: ammonium (NminNH4) and nitrate (NminNO3) generation and ammonium (Amp) and nitrate (NO3) content determination in microplate-format were done as described in Chapter 2.2.4.

8.1.2. Statistical analyses

The data were pretreated as described in Chapter 2.3. Data collected from the two remnants in same site, i.e. in L and Y, were averaged to avoid pseudo-replication which resulted in four true replicates, equivalent to sites.

Principal component analysis (PCA) using standardized data from the baseline data set were used to investigate the relationships among the original variables. The effects of treatment and site were analyzed using variation partitioning and distance-based redundancy analysis as described in Chapter 2.3. For the initial baseline data set, testing for the effects of treatment as a dummy factor was used to investigate the background differences among experimental plots that could confound the results. Only the models with significant effects of the experimental treatments are reported in detail.
Table 8.1. Mean values (±SD) of selected chemical properties of the soil in the study sites.

<table>
<thead>
<tr>
<th>Soil property (abbreviation)</th>
<th>Unit</th>
<th>Reference</th>
<th>C</th>
<th>L</th>
<th>P</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gravimetric moisture (M)</td>
<td>%</td>
<td>Burman et al. (1996)</td>
<td>24.54 ± 3.50</td>
<td>31.58 ± 4.10</td>
<td>27.12 ± 4.29</td>
<td>20.21 ± 4.03</td>
</tr>
<tr>
<td>Ammonium-N (A)</td>
<td>mg kg⁻¹</td>
<td>Searle (1984)</td>
<td>12.00 ± 3.46</td>
<td>18.50 ± 11.93</td>
<td>18.00 ± 18.17</td>
<td>35.70 ± 45.27</td>
</tr>
<tr>
<td>Exchangeable phosphorus - Colwell (P)</td>
<td>mg kg⁻¹</td>
<td>Rayment and Higginson (1992)</td>
<td>19.50 ± 5.23</td>
<td>20.30 ± 8.06</td>
<td>40.20 ± 13.85</td>
<td>14.60 ± 5.33</td>
</tr>
<tr>
<td>Exchangeable potassium - Colwell (K)</td>
<td>mg kg⁻¹</td>
<td>Rayment and Higginson (1992)</td>
<td>196.70 ± 63.60</td>
<td>209.10 ± 68.48</td>
<td>242.10 ± 83.71</td>
<td>150.45 ± 60.13</td>
</tr>
<tr>
<td>Sulphur (S)</td>
<td>%</td>
<td>Blair et al. (1991)</td>
<td>10.03 ± 1.35</td>
<td>10.07 ± 4.79</td>
<td>13.76 ± 5.65</td>
<td>19.88 ± 20.98</td>
</tr>
<tr>
<td>Organic carbon (OrgC)</td>
<td>%</td>
<td>Walkley and Black (1934)</td>
<td>8.76 ± 1.83</td>
<td>9.37 ± 1.17</td>
<td>9.39 ± 0.83</td>
<td>8.40 ± 1.77</td>
</tr>
<tr>
<td>Conductivity (Cond)</td>
<td>dS m⁻¹</td>
<td>Rayment and Higginson (1992)</td>
<td>0.10 ± 0.01</td>
<td>0.13 ± 0.04</td>
<td>0.13 ± 0.03</td>
<td>0.12 ± 0.07</td>
</tr>
<tr>
<td>pH in CaCl₂ (pH)</td>
<td></td>
<td>Rayment and Higginson (1992)</td>
<td>4.75 ± 0.15</td>
<td>4.50 ± 0.20</td>
<td>4.51 ± 0.31</td>
<td>4.64 ± 0.27</td>
</tr>
<tr>
<td>Total nitrogen – Kjedahl (TotN)</td>
<td>%</td>
<td>Rayment and Lyons (2011)</td>
<td>0.61 ± 0.15</td>
<td>0.78 ± 0.25</td>
<td>0.70 ± 0.17</td>
<td>0.52 ± 0.18</td>
</tr>
</tbody>
</table>

Table 8.2. Experimental treatments in the study of selected restoration practices in remnants of native vegetation in *Eucalyptus globulus* plantations in the south west of Western Australia. *- two plots per remnant with one remnant per site (sites C and P)/ or two remnants per site (sites L and Y)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glyphosate 0.72 g m⁻²</th>
<th>Harvest residue</th>
<th>Simazine 167 mg m⁻²</th>
<th>Replicate plots per site</th>
<th>Planted plots per site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-treated control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2/4*</td>
<td>1/2*</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>2/4*</td>
<td>1/2*</td>
</tr>
<tr>
<td>Mulch</td>
<td>-</td>
<td>√</td>
<td>-</td>
<td>2/4*</td>
<td>1/2*</td>
</tr>
<tr>
<td>Glyphosate + Mulch</td>
<td>√</td>
<td>√</td>
<td>-</td>
<td>2/4*</td>
<td>1/2*</td>
</tr>
<tr>
<td>Glyphosate + Simazine</td>
<td>√</td>
<td>-</td>
<td>√</td>
<td>2/4*</td>
<td>1/2*</td>
</tr>
</tbody>
</table>

194
For the baseline data set, the effects of treatment and site on soil properties (n = 40 observations) was tested. Using the end-point data set of this study, the effect of planting on soil properties and exotic ground cover measures was tested alongside the effects of the treatment and site (n = 40 observations). The effects of treatment on seedling growth and survival were tested separately using data collected from planted plots only (n = 20). Firstly, the effect of treatment as a single factor with five levels was tested, and when found to be significant, the analysis of the individual effects of the three restoration treatments, i.e. mulch, glyphosate and simazine, was conducted. A simultaneous analysis of these effects was impossible due to unbalanced design of the study. To overcome this issue, the data set was split into two subsets for analysis as follows: the effect of simazine was tested in a one-way design against glyphosate-only treatment as control, with n = 8 replicates each; the effect of glyphosate and mulching was tested in a two-way fully factorial design with n = 8 replicates each.

In addition, univariate analysis of variance (ANOVA) was done using the exotic ground cover and seedlings survival and growth data. The effects of planting and restoration treatments on exotic ground cover were tested in a two-way design with site as a blocking factor. The survival and growth of seedlings was investigated for the effects of restoration treatments with site as a blocking factor. The non-significant terms were removed to obtain parsimonious models. The quality of the models was determined by inspection of the residual plots and Tukey’s Honestly Significant Difference tests were used to correct for multiple comparisons.

8.2. Results

There were no differences in soil properties among plots before treatment application (Table 8.3). However, there was a significant effect of site on soil properties (Table 8.3), which was also evident in the PCA (Fig. 8.1). The soils from different sites varied especially in N-related properties such as total N, ammonium and nitrate content.
and N-mineralization potential (Fig. 8.1). The appearance of the experimental plots after application of treatments (Fig. 8.2) was similar within treatments across the sites, with non-treated control plots similar to the areas surrounding the experimental plots.

Table 8.3. Effects of experimental treatments on soil properties, exotic ground cover and planted seedlings survival and growth, detected by variation partitioning. Models focusing on the effects of experimental treatments independent of other factors are highlighted in bold.

<table>
<thead>
<tr>
<th>Response</th>
<th>Month&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Factor</th>
<th>Df</th>
<th>Adj R&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Pseudo-F</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil properties</td>
<td>0</td>
<td>Treatment* + Site</td>
<td>7</td>
<td>32</td>
<td>0.16</td>
<td>2.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment* - Site</td>
<td>4</td>
<td>32</td>
<td>0</td>
<td>1.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site - Treatment*</td>
<td>3</td>
<td>32</td>
<td>0.17</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Treatment + Site + Planting</td>
<td>8</td>
<td>31</td>
<td>0.12</td>
<td>1.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment - (Site+ Planting)</td>
<td>4</td>
<td>31</td>
<td>0.01</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site - (Treatment + Planting)</td>
<td>3</td>
<td>31</td>
<td>0.13</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Planting - (Treatment + Site)</td>
<td>1</td>
<td>31</td>
<td>0</td>
<td>0.91</td>
</tr>
<tr>
<td>Exotic ground cover and seedling survival and growth</td>
<td>11/10</td>
<td>Treatment + Site</td>
<td>7</td>
<td>12</td>
<td>0.26</td>
<td>1.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment - Site</td>
<td>4</td>
<td>12</td>
<td>0.13</td>
<td>1.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site – Treatment</td>
<td>3</td>
<td>12</td>
<td>0.19</td>
<td>2.25</td>
</tr>
</tbody>
</table>

* - dummy factor; <sup>a</sup> – model includes data from planted plots only; <sup>b</sup> - months after treatment application for soil properties and exotic ground cover / for planting for seedlings survival and growth data. Abbreviations: Df – degrees of freedom; M – model; R – residual; Adj – adjusted.

At 11 months after treatment application, the majority of the planted seedlings survived and had grown; however, the effect of planting or restoration treatments on soil properties was not significant (Table 8.3). Marked differences due to treatment were measured in seedling survival and growth at this point of the study, also when the exotic cover data from the planted plots was included in the response (Table 8.3). For clarity, only the latter model was investigated further in the db-RDA. A significant effect of site was detected in most of the models; however, this result was biased by the fact that the assumption of the homogeneity of the variance-covariance matrices was violated (Borcard et al. 2011) and is therefore not reported.
Figure 8.1. First two principal components of the analysis of soil properties before treatment application. The percentage of the variability in the data set explained by the principal components is given in parentheses. Symbols code for sites □ = C; ● = L; ▲ = P; □ = Y; continuous lines represent component loadings. Abbreviations: M- moisture, NitN- nitrate-N, A- ammonium-N, P- phosphorus, K- potassium, S- sulphur, OrgC- organic carbon, TotN- total nitrogen, Cond- conductivity, pH- pH in CaCl2, FH- fluorescein diacetate hydrolysis activity, Amp- ammonium by microplate method; NminNH4- N-mineralization potential.

Separate univariate analyses for each of the plant species revealed that the differences in seedling survival and growth due to restoration treatments were not consistent across species (Fig. 8.3). Of the measures influenced by the restoration treatments, the survival and growth of *A. pulchella* responded favourably to the glyphosate with simazine treatment, with growth benefiting also from glyphosate with mulch treatment. Survival, but not growth of *B. grandis* was higher in mulched plots treated with glyphosate; glyphosate also increased the height, but not survival of *A. flexuosa* and *K. prostrata* (Fig. 8.3).
Figure 8.2. Effects of selected restoration treatments at 4 months after application at site P. The appearance of plots was representative across the three sites, and was similar for planted and non-planted treatments: (A) non-treated control; (B) mulching with harvest residue; (C) mulching with harvest residue after glyphosate application; (D) combined simazine and glyphosate treatment. Glyphosate treatment alone was identical to (D) at this time (not shown).
A significant effect of both planting and treatment on exotic ground cover was measured, and was investigated further in an analysis of the split exotic ground cover data (see section 8.2.3). Significant effects of planting and glyphosate were found in the two-way design analysis and of simazine in the one-way design analysis (Table 8.4).

### Table 8.4. Effects of experimental treatments on exotic ground cover (EC) and planted seedlings growth and survival (SGS) at 11 months after treatment application, i.e. 10 months after planting, tested by variation partitioning. Bold characters highlight values significant at the level of α=0.05.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Response</th>
<th>Df Model</th>
<th>Df Residual</th>
<th>Adjusted R²</th>
<th>Pseudo-F</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planting</td>
<td>EC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>25</td>
<td>0.04</td>
<td>2.5</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>SGS</td>
<td></td>
<td></td>
<td></td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>Glyphosate</td>
<td>EC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>25</td>
<td>0.02</td>
<td>1.89</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td>SGS&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
<td>10</td>
<td>0.11</td>
<td>2.47</td>
<td>0.008</td>
</tr>
<tr>
<td>Mulch</td>
<td>EC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>25</td>
<td>0.00</td>
<td>0.91</td>
<td>0.530</td>
</tr>
<tr>
<td></td>
<td>SGS&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
<td>10</td>
<td>0.03</td>
<td>1.39</td>
<td>0.180</td>
</tr>
<tr>
<td>Simazine*</td>
<td>EC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>10</td>
<td>0.08</td>
<td>2.25</td>
<td>0.034</td>
</tr>
<tr>
<td></td>
<td>SGS&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
<td>3</td>
<td>0.09</td>
<td>1.44</td>
<td>0.260</td>
</tr>
</tbody>
</table>

*<sup>a</sup> n = 20 per group; <sup>b</sup> n = 8 per group; <sup>c</sup> n = 4 per group; * tested against glyphosate — only as control treatment. Abbreviations: Df — degrees of freedom; na — not available.

Univariate analyses revealed that only the glyphosate with simazine treatment significantly decreased exotic grass cover as compared to control (Fig. 8.4 A), with no effects on exotic broadleaf cover (Fig. 8.4 B). Planting affected the average and maximum height of the exotic ground cover, which was higher in the planted plots; however, the effects on maximum height were site-dependent (Fig. 8.4 C and D).
Figure 8.3. Effects of restoration treatments on survival (A) and growth (B) of seedlings of six local native plant species at 10 months after planting. Error bars denote standard deviation of the mean; differences from the respective non-treated controls, significant at the level of $\alpha = 0.05$, are marked by asterisk (*).
The first two canonical axes explained a marked proportion of the variation accounted for by the model obtained in the db-RDA of the exotic ground cover and seedling survival and growth data at 11 months after treatment application (Table 8.5). The model revealed clear separation of the glyphosate treatments from control along the first canonical axis (CAP1) (Fig. 8.5 A). The differences among treatments, excluding the control, were evident along the second canonical axis (CAP2) (Fig. 8.5 A). All treatments except the control reduced exotic grass cover, while broadleaf cover was higher in the mulched-only plots and lower in glyphosate-simazine treated plots (Fig. 8.5). At 10 months after planting, herbicide application increased the survival and growth of most of the seedlings; however, species-specific responses to the experimental treatments were obvious (Fig. 8.5). The survival of *E. marginata*, *A. flexuosa* and *B. grandis* was higher in the mulched-only plots, while the survival and growth of *A. pulchella* was highest in the glyphosate-simazine treated plots (Fig. 8.5).

**Table 8.5.** Significance of the canonical axes (CAPs) in the distance-based redundancy analysis of the effects of experimental treatments on exotic ground cover and planted seedlings survival and growth at 10 months after planting (data from planted plots only). Bold characters highlight values significant at the level of $\alpha = 0.05$.

<table>
<thead>
<tr>
<th>CAP</th>
<th>Degrees of freedom</th>
<th>Variance</th>
<th>Pseudo-F</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.020</td>
<td>3.32</td>
<td>0.001</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.013</td>
<td>2.08</td>
<td>0.007</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0.006</td>
<td>0.93</td>
<td>0.57</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0.003</td>
<td>0.56</td>
<td>0.95</td>
</tr>
<tr>
<td>Residual</td>
<td>12</td>
<td>0.074</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 8.4. Effects of planting and restoration treatments on exotic ground cover measures: (A) percentage exotic grass cover; (B) percentage exotic broadleaf cover; (C) average and (D) maximum exotic plant cover. Error bars denote standard deviation of the mean; same letters denote no significant differences at the level of $\alpha = 0.05$. 
Figure 8.5. Effect of experimental treatments on exotic ground cover and seedlings survival and growth at 10 months after planting, detected by partial distance-based redundancy analysis. Weighted averages (“wa”) scores of the samples (scaling 1) (A) and of the original variables (scaling 2) (B) for the first two canonical axes (CAPs) are shown. Experimental treatments are coded by symbols: ☐- Non-treated control; ▲ - Mulching with harvest residue; ● - Glyphosate; ■ - Glyphosate + Simazine; △ - Glyphosate + Mulching with harvest residue. Dashed lines represent distances from the group centroids; continuous ellipses represent the values of one standard deviation from the group centroids; the percentage of the variability in the db-RDA model explained by the CAPs is given in parentheses. Abbreviations: gcov- percentage of ground covered by exotic grasses, bcov- percentage of ground covered by broadleaf exotic species, mht- maximum height of exotic ground cover, aht- average height of exotic ground cover; seedling measures: *E. marginata* survival (sja) and average height (ahja), *K.prostrata* survival (ske) and average height (ahke), *H. pungens* survival (she) and average height (ahhe), *A. flexuosa* survival (sag) and average height (ahag), *B.grandis* survival (sba) average height (ahba), *A. pulchella* survival (sac) and average height (ahac).
8.3. **Conclusions**

A decrease in exotic grass cover, caused by all restoration treatments investigated in the current study, coincided with an increase in survival and growth of planted seedlings; however, plant species – specific responses to the treatments were also observed. For example, *A. pulchella* was the only species that benefited from the simazine treatment, while mulching with plantation harvest residue improved survival and growth of *A. flexuosa* and jarrah seedlings. A more thorough investigation of treatments reducing exotic ground cover would be required in order to elucidate their effects on plants used in restoration. This would increase chances of success of revegetation protocols involving a mixture of native plant species.