Diversity, Functioning and Stability in Microbial Communities

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Doctor of Philosophy

Hawkesbury Institute for the Environment
University of Western Sydney

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or part, for a degree at this or any other institution.

Signature: ……………………………….. Date:………………

Federica Colombo
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Table of Contents

CHAPTER 1: General introduction ............................................................... - 1 -
  1.1. The importance of terrestrial microbial diversity.............................. - 2 -
  1.2. Biodiversity and ecosystem functioning ....................................... - 4 -
       1.2.1. Biodiversity components and their relationship with ecosystem
              functioning ........................................................................... - 6 -
       1.2.2. Impact of biodiversity loss on ecosystem functioning ............. - 16 -
  1.3. Ecological Sustainability ................................................................... - 19 -
       1.3.1. Defining ecosystem sustainability as ecosystem stability ........... - 19 -
       1.3.2. Stability in microbial communities ....................................... - 21 -
       1.3.3. Biodiversity-stability relationship ....................................... - 22 -
  1.4. Aims of this study ............................................................................ - 25 -

CHAPTER 2: Microbial diversity loss and functional stability to disturbance: dilution-to-
extinction experiment ........................................................................ - 27 -
  2.1. Introduction ...................................................................................... - 28 -
  2.2. Materials and methods ................................................................. - 30 -
       2.2.1. Soil sampling and soil characteristics ...................................... - 30 -
       2.2.2. Preparation of soil extract media (SEM) .................................. - 31 -
       2.2.3. Extraction and dilution of soil microbial community ............... - 31 -
       2.2.4. Biomass recovery monitoring ................................................. - 33 -
       2.2.5. Determination of relative bacterial diversity and richness ........ - 35 -
       2.2.6. Functional measurements ....................................................... - 38 -
       2.2.7. Disturbance and measurement of stability ............................... - 39 -
       2.2.8. Statistical methods .................................................................. - 41 -
  2.3. Results ............................................................................................ - 41 -
       2.3.1. Bacterial biomass recovery ...................................................... - 41 -
       2.3.2. Effects of dilution on bacterial diversity ................................... - 43 -
       2.3.3. Effects of dilution on function ................................................ - 45 -
       2.3.4. Bacterial diversity and richness: relationship with functioning .. - 47 -
       2.3.5. Resistance to disturbance along a diversity gradient ................... - 50 -
       2.3.6. Resilience to disturbance along a diversity gradient ................. - 52 -
  2.4. Discussion ....................................................................................... - 54 -
4.2.5. Statistical methods .................................................................................. 111
4.3. Results ........................................................................................................ 113
  4.3.1. Soil properties ................................................................................ 113
  4.3.2. Bacterial community composition, structure and diversity ............... 114
  4.3.3. Microbial abundances ................................................................ 120
  4.3.4. Potential enzyme activity .................................................................. 121
  4.3.5. Community respiration and substrate-induced respiration ............... 123
  4.3.6. Relationship between microbial communities and soil functions ....... 125
  4.3.7. Effects of forest management on soil functioning .............................. 131
4.4. Discussion ................................................................................................ 134
  4.4.1. Effects of forest management practices on soil microbial community structure... ............................... 134
  4.4.2. Relationship between microbial communities and soil functions ..... 136
  4.4.3. Effects of forest management practices on soil ecosystem functioning...... 138
4.5. Conclusions and future directions .......................................................... 141

CHAPTER 5: Microbial diversity and functional stability in natural soil communities ....... 144
  5.1. Introduction ............................................................................................ 145
  5.2. Materials and Methods ........................................................................ 146
  5.2.1. Sites description and soil sampling ..................................................... 146
  5.2.2. Soil microcosms ............................................................................... 146
  5.2.3. Disturbance treatment ..................................................................... 147
  5.2.5. Molecular analyses ......................................................................... 147
  5.2.6. Functional measurements ................................................................ 148
  5.2.7. Soil physicochemical analyses ......................................................... 148
  5.2.8. Statistical methods ......................................................................... 148
5.3. Results ....................................................................................................... 150
  5.3.1. Effects of disturbance on microbial communities .............................. 150
  5.3.2. Stability of soil functions in relation to disturbance ......................... 157
  5.3.3. Relationship between microbial communities and functional stability ... 164
  5.3.4. Effects of forest management on the stability of soil functions .......... 174
5.4. Discussion ................................................................................................ 179
  5.4.1. Effects of disturbance on bacterial diversity and functioning ............. 179
  5.4.2. Relationship between bacterial communities and stability ............... 181
  5.4.3. Effects of forest management practices on soil functional stability ...... 185
5.5. Conclusions and future directions

CHAPTER 6: General discussion

References

Appendix

APPENDIX A: Selection of temperature and duration of heat shock applied to measure stability to disturbance

APPENDIX B: Biomass recovery in soil extract medium experiments

APPENDIX C: Optimisation of substrate concentration for potential enzyme assays at Moora field site

APPENDIX D: Optimisation of substrate concentration for potential enzyme assays at HFE experiment

APPENDIX E: Functional measurements used for the calculation of resistance and resilience indexes

- 187 -

- 188 -

- 202 -

- 230 -

- 231 -

- 236 -

- 237 -

- 238 -

- 239 -
List of Tables

**Table 1.1** Components and definitions of biodiversity and ecosystem functioning .................................................. (Christensen et al., 1996, Hooper et al., 2005) (Christensen et al., 1996, Hooper et al., 2005)................................. 5

**Table 2.1** Oligonucleotides used for the amplification of bacterial ribosomal DNA in qPCR reactions................................................................................................................................. 34

**Table 2.2** Oligonucleotides used for the amplification of bacterial ribosomal DNA in PCR reactions for T-RFLP................................................................................................................................................... 35

**Table 3.1** List of treatments at the Moora pasture cropping site.................. 68

**Table 3.2** Oligonucleotides used for the amplification of bacterial and fungal ribosomal DNA in qPCR reactions................................................................................................................................. 70

**Table 3.3** Substrates used in extracellular enzyme assays.......................... 71

**Table 3.4** The 11 C substrates used in the MicroResp™ assay, their classification and number of carbon (C) and nitrogen (N) atoms in their molecule.......................... 74

**Table 3.5** Mean values of soil pH, soil moisture content, total carbon, total nitrogen, phosphate concentrations (PO₄³⁻), ammonium concentrations (NH₄⁺), nitrate concentrations (NO₃⁻) across different treatments.................................................................................................................................................. 80

**Table 3.6** Results of the multiple linear regressions constructed with each class of variables, for each soil function investigated................................................................. 94

**Table 3.7** Model fit for the final model that best explains variation of each function, for models in which the variables were dropped one by one, and for intercept-only models................................................................................................................................................. 95

**Table 3.8** Location and vegetation description of natural forest sites........... 109
Table 4.2 Mean values of soil pH, soil moisture content, total carbon, total nitrogen, phosphate concentrations (PO$_4^{3-}$), ammonium concentrations (NH$_4^+$), nitrate concentrations (NO$_3^-$) across different forest-management treatments..................- 114 -

Table 4.3 Results of the multiple linear regressions constructed with each class of variables, for each ecosystem function investigated.................................................................- 127 -

Table 4.4 Model fit for the final model which best explains variation of each function, for models in which the variables were dropped one by one, and for intercept-only models.........................................................................................................- 128 -

Table 5.1 Results of the multiple linear regressions constructed with each class of variables, for the resistance of each ecosystem function investigated.........................- 166 -

Table 5.2 Model fit for the final model which best explains variation of the resistance of each ecosystem function, for models including the significant variables, and for intercept-only models........................................................................................................- 167 -

Table 5.3 Results of the multiple linear regressions constructed with each class of variables, for the resilience of each ecosystem function investigated.......................- 171 -

Table 5.4 Model fit for the final model which best explains variation of the resilience of each ecosystem function, for models in which the variables were dropped one by one, and for intercept-only models........................................................................................................- 172 -
List of Figures

**Figure 1.1** Patterns of the biodiversity-ecosystem functioning (BEF) relationship............................................................................................................................................- 8 -

**Figure 1.2** Relationship between the degree of evenness of a community and its stability when undergoing a perturbation.............................................................................................................................- 15 -

**Figure 1.3** Graph illustrating the effects of disturbance on resistance and resilience of ecosystem functioning........................................................................................................................................- 20 -

**Figure 2.1** Schematic representation of the design used to test the relationship between biodiversity, function and stability..........................................................................................................................- 32 -

**Figure 2.2** Bacterial biomass recovery at different dilution levels: data of copies number ml⁻¹ of bacterial 16S rRNA gene..........................................................................................................................- 42 -

**Figure 2.3** Effects of dilution on relative OTU richness, diversity, and evenness based on T-RFLP data......................................................................................................................................................- 44 -

**Figure 2.4** Respiration rates measured after biomass recovery along the dilution gradient for: control (i.e. not amended), glucose, and lignin..............................................................................................- 46 -

**Figure 2.5** Relationship between bacterial diversity and respiration. Bacterial diversity is estimated by T-RFLP analysis..........................................................................................................................- 48 -

**Figure 2.6** Relationship between bacterial OTU richness and respiration. Bacterial diversity is estimated by T-RFLP analysis..........................................................................................................................- 49 -

**Figure 2.7** Resistance indices calculated on respiration rates data collected before and after shock along the dilution gradient for control, glucose, and lignin..................................................................- 51 -

**Figure 2.8** Resilience indices calculated on respiration data collected before shock, after shock, and four days after shock along the dilution gradient for control, glucose, and lignin..........................................................................................................................- 53 -
Figure 3.1 Ordination plot of the first two axes produced by principal coordinates analyses of Manhattan distances of bacterial community composition, based on amplicon 16S rRNA gene data.................................................................- 82 -

Figure 3.2 Ordination plot of the first two axes produced by redundancy analysis based on Manhattan distances. Effects of edaphic variables on bacterial community composition, based on amplicon 16S rRNA gene data.............................................- 83 -

Figure 3.3 Effects of treatments on OTU richness, Simpson’s index of diversity, and Pielou’s index of evenness.................................................................- 85 -

Figure 3.4 Bacterial and fungal abundances across different treatments............- 87 -

Figure 3.5 Potential activity of β-glucosidase, N-acetyl-glucosaminidase, and acid phosphatase across different treatments.................................................................- 89 -

Figure 3.6 Mean respiration rates as measured for the control (i.e. no substrate added) and sum of respiration responses to the 11 substrates used in the MicroResp™ assay across different treatments.............................................- 91 -

Figure 3.7 Bivariate correlations between significant variables in the final model and β-glucosidase, N-acetyl-glucosaminidase, acid phosphatase, and basal respiration .............................................................................................................- 96 -

Figure 4.1 Illustration of the relationship tested by structural equation modelling. The model represents direct, causal relationships among variables.................- 113 -

Figure 4.2 Ordination plot of the first two axes produced by principal coordinates analyses of Bray–Curtis distances of bacterial community composition, and relative abundance of phyla based on amplicon 16S rRNA gene data.............................................- 116 -

Figure 4.3 Ordination plot of the first two axes produced by redundancy analysis based on Bray–Curtis distances. Effects of edaphic variables on bacterial community composition, based on amplicon 16S rRNA gene data.............................................- 117 -

Figure 4.4 Effects of forest management treatments on OTU richness, Simpson’s index of diversity, and Pielou’s index of evenness calculated on sequencing data.............................................................................................................- 119 -
Figure 4.5 Bacterial and fungal abundances across different treatments............- 120 -

Figure 4.6 Potential activity of β-glucosidase, N-acetyl-glucosaminidase, and acid phosphatase in the different soil treatments.................................................................- 122 -

Figure 4.7 Sum of respiration responses to the 11 substrates used in the MicroResp™ assay in the different treatments..............................................- 123 -

Figure 4.8 Mean respiration rates as measured for the control (i.e. no substrate added) in the MicroResp™ assay in the different treatments.........................- 124 -

Figure 4.9 Redundancy analysis of carbon source utilisation patterns, measured by MicroResp™.........................................................................................- 125 -

Figure 4.10 Bivariate correlations between single variables in the best-fitting model and the activities of β-glucosidase, N-acetyl-glucosaminidase, acid phosphatase, and the rates of basal respiration and total substrate responses........................................- 130 -

Figure 4.11 Results from structural equation modelling on the predictors of β-glucosidase activity, N-acetyl-glucosaminidase activity, acid phosphatase activity........................................................................................................- 132 -

Figure 4.12 Results from structural equation modelling on the predictors of basal respiration and sum of substrate responses.......................................................- 133 -

Figure 5.1 Effects of heat disturbance on OTU richness, Simpson’s index of diversity, and Pielou’s index of evenness calculated on T-RFLP profiles measured two days after disturbance..................................................................................- 152 -

Figure 5.2 Effects of heat disturbance on OTUs richness, Simpson’s index of diversity, and Pielou’s index of evenness calculated on T-RFLP profiles measured 30 days after disturbance................................................................................- 153 -

Figure 5.3 Bacterial abundances in the soils incubated continuously at room temperature and in those that were subjected to heat disturbance, as measured after two days and 30 days....................................................................................- 155 -
Figure 5.4 Fungal abundances in the soils incubated continuously at room temperature and in those that were subjected to heat disturbance, as measured after two days and 30 days.

Figure 5.5 Resistance indices calculated on potential enzyme activity of β-glucosidase, N-acetyl-glucosaminidase, and acid phosphatase, collected one day after shock in disturbed and undisturbed soils.

Figure 5.6 Resilience indices calculated on potential enzyme activity of β-glucosidase, N-acetyl-glucosaminidase, and acid phosphatase, measured one month after shock in disturbed and undisturbed soils.

Figure 5.7 Resistance indices calculated on basal respiration rates and sum of substrate response rates measured one day after shock in disturbed and undisturbed soils.

Figure 5.8 Resilience indices calculated on basal respiration rates and sum of substrate respiration responses rates measured a month after shock in disturbed and undisturbed soils.

Figure 5.9 Bivariate correlations between single variables in the full model and resistance index of N-acetyl-glucosaminidase, acid phosphatase activity, and sum of respiration responses.

Figure 5.10 Bivariate correlations between single variables in the full model and resilience index of β-glucosidase, acid phosphatase, N-acetyl-glucosaminidase activity, and basal respiration and sum of respiration responses.

Figure 5.11 Results from structural equation modelling on the predictors of resistance indices of N-acetyl-glucosaminidase activity, acid phosphatase activity, and sum of respiration responses.

Figure 5.12 Results from structural equation modelling on the predictors of resilience indices of β-glucosidase activity, N-acetyl-glucosaminidase activity, acid phosphatase activity, basal respiration, and sum of respiration responses.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AIC</td>
<td>Akaike information criterion</td>
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<tr>
<td>AICc</td>
<td>Akaike information criterion corrected for small sample size</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>AP</td>
<td>acid phosphatase</td>
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<td>BEF</td>
<td>biodiversity-ecosystem functioning relationship</td>
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<td>BG</td>
<td>β-glucosidase</td>
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<td>bp</td>
<td>base pairs</td>
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<td>C</td>
<td>carbon</td>
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<td>ca</td>
<td>circa</td>
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<td>cm</td>
<td>centimetre</td>
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<td>CO₂</td>
<td>carbon dioxide</td>
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<td>dbRDA</td>
<td>distance-based redundancy analysis</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>E</td>
<td>east</td>
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<td>e.g.</td>
<td><em>exempli gratia</em> (example)</td>
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<td>g</td>
<td>gram</td>
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<td>h</td>
<td>hour</td>
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<tr>
<td>ha</td>
<td>hectares</td>
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<td>HFE</td>
<td>Hawkesbury forest experiment</td>
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<tr>
<td>i.e.</td>
<td><em>id est</em> (that is)</td>
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<tr>
<td>kg</td>
<td>kilogram</td>
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<td>m</td>
<td>metre</td>
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<td>mEq</td>
<td>milliequivalent</td>
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<td>min</td>
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<td>mM</td>
<td>millimolar</td>
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<tr>
<td>MUB</td>
<td>4-Methylumbelliferone</td>
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<td>N</td>
<td>nitrogen</td>
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<td>n</td>
<td>number</td>
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<td>NAG</td>
<td>N-acetyl-glucosaminidase</td>
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<td>Abbr.</td>
<td>Term</td>
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<tr>
<td>NAR</td>
<td>Northern Agricultural Region</td>
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<tr>
<td>ng</td>
<td>nanogram</td>
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<td>nanometre</td>
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<tr>
<td>ns</td>
<td>not significant</td>
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<tr>
<td>NSW</td>
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<tr>
<td>OTU</td>
<td>operational taxonomic unit</td>
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<td>P</td>
<td>phosphorus</td>
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<td>PCA</td>
<td>principal components analysis</td>
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<td>polymerase chain reaction</td>
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<td>PerMANOVA</td>
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<td>pH</td>
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<td>qPCR</td>
<td>quantitative real-time polymerase chain reaction</td>
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<td>r.p.m.</td>
<td>rounds per minute</td>
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<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
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<td>SEM</td>
<td>soil extract medium</td>
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<td>sp.</td>
<td>Species (singular)</td>
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<td>spp.</td>
<td>Species (plural)</td>
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<td>T-RFLP</td>
<td>Terminal restriction fragment length polymorphism</td>
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<td>TRFs</td>
<td>Terminal restriction fragments</td>
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<td>unit</td>
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<td>UV</td>
<td>ultraviolet</td>
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<td>WA</td>
<td>Western Australia</td>
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Abstract

Biodiversity loss is among the major drivers of ecosystem change. Understanding the consequences of loss of biodiversity on the functioning of the ecosystems is of paramount importance in order to assess the sustainability of a system. Ecosystem stability is a measure of sustainability and it is defined as the degree of resistance and resilience of a system to perturbations. Microbes are the most abundant organisms on Earth and they are responsible for many ecologically important ecosystem processes. Despite this, the knowledge of the role of their diversity in ecosystem functioning and functional stability is still incomplete. This research investigated the relationship between bacterial diversity and functioning (BEF) and stability in soil ecosystems in order to better understand the consequences of decline in microbial diversity on ecosystem sustainability.

Firstly, the soil microbial community was experimentally manipulated using the dilution-to-extinction approach in a soil extract medium microcosms experiment to analyse the consequences of microbial diversity loss on general and specific functions and their stability to a heat disturbance. Community respiration and the utilisation of compounds of different complexity (i.e. glucose and lignin) were used as functional parameters. Terminal restriction fragment length polymorphism (T-RFLP) analysis revealed that a reduction in richness of 77% led to a significative decrease in respiration rates in aggregate function (i.e. community respiration) and specific functions, but did not affect general functions. A positive linear correlation was found between bacterial richness and community respiration and lignin-induced respiration, but it was not possible to identify a clear relationship with the utilisation of glucose. Bacterial communities with lower diversity showed lower functional
stability to heat disturbance. These results indicate that both general and specific
functions depend on bacterial diversity and a loss of diversity in soil systems has the
potential to affect their functional stability.

The BEF under environmental conditions was then investigated in agricultural and
forest ecosystems by means of field studies. Finally, I investigated the functional
stability of forest soils with naturally different levels of bacterial diversity following
a transient heat disturbance in a soil microcosm experiment. Microbial processes
involved in nutrients cycling were analysed by means of potential enzyme activity,
basal respiration and substrate-induced respiration. The diversity and composition of
bacterial communities were investigated by means of Illumina MiSeq sequencing of
the 16S rRNA gene. The relationship between bacterial diversity components (i.e.,
diversity, richness, composition) and soil processes and functional stability was
investigated by means of stepwise multiple regressions examining the explanatory
power of microbial community variables and environmental properties.

In the agricultural system investigated, the combination of different perennial grasses
with crop, under different nitrogen regimes did not produce sharp differences in
bacterial communities variables and no correlation between microbial communities
and soil functioning was found. The main predictors of soil processes were abiotic
factors, suggesting that the soil environment had a stronger influence on the
functional properties than the biotic component in these soils.

In a forest plantation experiment, the application of irrigation and fertilisation
influenced bacterial community composition and diversity. The functioning of forest
soils was predicted by bacterial richness and abundance, and by edaphic factors.
Bacterial richness and abundance, and soil functions involved in nutrient cycling
were linked by significant linear relationship. Changes in microbial diversity had functional consequences for soil processes, showing that even a moderate loss (circa 20%) in biodiversity can have important effects on ecosystem functioning.

The functional stability of forest soils was estimated by evaluating resistance and resilience to heat disturbance of soil respiration and potential enzymes activity. The stepwise multiple regressions. The resistance and resilience of all functions analysed were predicted by bacterial diversity components, demonstrating that bacterial communities are important drivers of soil functional stability in the forest ecosystem analysed.

The results of this work highlight that declining microbial diversity has direct consequences for functioning and functional stability to disturbance of soil ecosystems. These findings suggest that the functional redundancy (i.e. when several species in a system carry out the same function) of microbial communities may be not as prevalent as generally thought, and hence preserving the microbiota might be as important as preserving the diversity of higher organisms, to ensure the sustainability of the ecosystems. This work also suggests that disruptive land management practices, such as agricultural ones, can influence the BEF in soil ecosystems through habitat homogenisation and modification of biogeochemical cycles.
CHAPTER 1

General introduction
1.1. The importance of terrestrial microbial diversity

Global biodiversity is changing at an unprecedented rate in response to several human-induced environmental modifications, such as variations in nutrient availability, land use, atmospheric composition, climate and the introduction of exotic species (Chapin et al., 2000, Grace et al., 2007). It has been reported that high levels of species loss affect ecosystem functions, such as productivity and decomposition, to the same extent as other major drivers of environmental change, such as ozone, acidification, elevated CO$_2$ and nutrient pollution (Hooper et al., 2012). The proper functioning of an ecosystem depends on the quantity and quality of the species present and, hence, loss of biodiversity is likely to impact the capacity of the environment to provide goods and services fundamental to human life. Therefore, understanding the relationship between biological diversity and ecosystem functioning (BEF) plays a paramount role in predicting the consequences of global change on natural systems, and for the assessment of the ecological sustainability of an ecosystem.

Consequently, the BEF relationship has emerged as a major scientific issue and it has been the centre of intense study. This research effort provided the unequivocal evidence that biodiversity enhances the efficiency of ecosystems to capture resource, produce biomass, and recycle nutrients (Cadotte et al., 2009, Flynn et al., 2011, Cardinale et al., 2012). How biodiversity contributes to the stability and maintenance of ecosystem processes in the face of anthropogenic perturbations is a fundamental question in environmental science. However, the majority of research on this topic has left aside the microscopic components of the ecosystems and, as a result, our
understanding of the functional consequences of microbial diversity loss is less well
developed.

One of the biggest challenges in contemporary ecology is the understanding the
ecology of microorganisms. Prokaryotes are undoubtedly the most numerous
organisms, and have been estimated to count between 4 and 6 x $10^{30}$ cells in
terrestrial ecosystems (Whitman et al., 1998). They carry out processes vital for the
whole ecosystem, such as degradation of organic matter, cycling of nutrients,
sequestration of carbon, production and consumption of trace gases, and degradation
of pollutants (Falkowski et al., 2008). They are essential components of
biogeochemical cycles (Falkowski et al., 2008), and represent approximately half of
the total carbon contained in living organisms (Shively et al., 2001). Microorganisms
affect atmospheric gas concentrations (Bardgett et al., 2008) and influence the
fertility of soils and oceans (Falkowski et al., 2008). In fact, half of the total carbon
emitted to the atmosphere every year from terrestrial ecosystems is attributed to
heterotrophic soil microorganisms (Reay and Cin, 2007). They contribute greatly to
net carbon exchange through decomposition and heterotrophic respiration and
through their interactions with plants and by modifying nutrient availability in the
soil (van der Heijden et al., 2008). Despite their importance, our knowledge about
the biodiversity of microorganisms and their role in the ecosystem functioning is still
incomplete, at least compared to what is known about aboveground diversity and
functioning. Since many microbial processes are essential for ecosystem
sustainability (e. g. biogeochemical cycling), understanding the factors that control
these processes is crucial. The current absence of information is due not only to the
huge genetic, metabolic and specific diversity of microorganisms, but also to the
capacity to culture only from 1 to 10 % of them (Amann et al., 1995, Pace, 1997, Sait et al., 2002, Joseph et al., 2003).

Significant research has been carried out on plant and animal communities and their relationships with ecosystem functioning, establishing a body of theory (Hillebrand and Matthiessen, 2009, Cardinale et al., 2012, Hooper et al., 2012). The same cannot be said for the microbial realm, where the development of theory is lacking and the role of microbial communities in the regulation of ecosystem processes is not well studied. The application of cultivation-independent molecular techniques has made possible new and more comprehensive observations of microorganisms in nature, but we still lack theoretical tools to detect underlying principles and mechanisms (Prosser et al., 2007). The challenge now facing microbial ecologists is, hence, to develop an appropriate theoretical framework to demonstrate its general applicability to microbial ecology. This would provide opportunities for ecologists to test the true generality of ecological principles, and would greatly increase our understanding of ecosystems, allowing more effective management and conservation of the environment and its resources.

1.2. **Biodiversity and ecosystem functioning**

Much of the research on biodiversity-functioning relationship in terrestrial environments has been mainly focused on aboveground diversity and, therefore, most of the principles overviewed here are derived from plant ecology. Belowground biodiversity effects on the functioning of terrestrial ecosystems are only starting to be identified. Whether those principles can be applied to other systems (e. g. soil
microbial diversity) remains to be investigated. Definitions of the concepts of biodiversity and ecosystem functioning and their components are listed in Table 1. Microbial ecosystem function typically relates to biogeochemical cycling, such as carbon mineralisation and nitrification rates, given its importance for the ecosystems and the central role played by microorganisms in nutrient cycling. Many studies investigating the BEF relationship in microbial communities measured processes such as respiration (e.g. Bell et al., 2005, Wertz et al., 2007), nitrification and denitrification rates (e.g. Wertz et al., 2006, Philippot et al., 2013), decomposition of organic compounds (e.g. Wohl et al., 2004, Tiunov and Scheu, 2005) and of pesticides (e.g. Singh et al., 2014) and enzyme activities (e.g. Riah-Anglet et al., 2015).

**Table 1.1** Components and definitions of biodiversity and ecosystem functioning (Christensen et al., 1996, Hooper et al., 2005).

<table>
<thead>
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<th>Components of Biodiversity</th>
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<tr>
<td><strong>Richness:</strong> the number of species present in a system</td>
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<tr>
<td><strong>Evenness:</strong> the relative abundance of species</td>
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<tr>
<td><strong>Composition:</strong> the identity of species present</td>
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<tr>
<td><strong>Functional traits:</strong> attributes of species that influence ecosystem properties or species’ responses to environmental conditions</td>
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**Ecosystem functioning:** the biogeochemical activities of an ecosystem or the flow of materials, such as nutrients, water, atmospheric gasses, and processing of energy

- **Ecosystem services:** series of benefits that ecosystems provide to humanity, including the regulation of processes (e.g. nutrient cycling, production of biomass, removal of greenhouse gases such as carbon dioxide from the atmosphere, maintaining hydrologic cycles)
- **Ecosystem properties:** pools of materials, such as carbon or organic matter, and rates of processes, such as fluxes of materials and energy among compartments
- **Ecosystem goods:** those ecosystem properties that have a direct market value
A complete understanding of the relationship between terrestrial biodiversity and ecosystem processes is of crucial importance in order to link above- and belowground components and hence soil diversity and ecosystem functioning to better predict consequences of the loss of biodiversity on a global scale.

1.2.1. Biodiversity components and their relationship with ecosystem functioning

1.2.1.1. Species Richness

Much ecological research has focused on the relationship between aboveground species richness and ecosystem functioning, leading to the construction of several theories (Figure 1.1). This relationship can be positive in some cases, due to two main mechanisms (Tilman et al., 1997a, Loreau, 2000). First, only one or a few species might have a large effect on a given ecosystem property. Therefore, increasing species richness increases the probability that those key species are present. This is known as the sampling effect or selection probability effect (Figure 1.1). According to this principle, higher richness increases the chance, for instance, that a superior competitor would be present and interspecific interactions would cause it to become dominant. Dominant species traits, therefore, would become the major determinant of ecosystem functioning. Second, species or functional richness could increase ecosystem properties through positive interactions among species, such as facilitation, or by occupying complementary ecological niches, for example by feeding on different resources. This mechanism is primarily due to complementarity and facilitation (Figure 1.1). Complementarity results from reduced interspecific competition through niche partitioning. When species use different resources or the same resource but at different times, the overall use of resources of a
community is more efficient (Vandermeer, 1989). In this way, increasing functional richness should lead to higher productivity and decreased loss of resources. Facilitative interactions can occur when certain species moderate harsh environmental conditions or provide a critical resource for other species (Fowler, 1986, Chapin et al., 1994), and result as well in an overall increase of process rates. Sampling and complementarity effects are not exclusive, but they can operate simultaneously (Fargione et al., 2007). The most commonly hypothesised pattern is a saturating response of ecosystem functions to increasing species richness due to functional redundancy, i. e. the capability of several species to perform the same function (Figure 1.1). As the richness increases, in fact, it is more likely that the same ecosystem function is carried out by multiple species. In plant ecology studies, the response of plant biomass to species richness has been characterised as saturating (Cardinale et al., 2011, Schnitzer et al., 2011) or increasing in a decelerating manner (Hector et al., 1999, Tilman et al., 2001, Marquard et al., 2009), indicating that adding more species does not produce a further increase in function. Nonetheless, the saturation of functioning with increasing species number has been challenged by the analysis of the shape of the BEF relationship in long-term plant experiments (Reich et al., 2012). Over time, the effects of plant diversity on biomass productivity increased and became less saturating, due to the accumulating effects of complementarity of resource use (Reich et al., 2012). Hence, diversity loss might have greater negative impacts on ecosystem functioning on a longer time scale than has been suggested by short-term experiments.
Figure 1.1 Patterns of the biodiversity-ecosystem functioning (BEF) relationship (Adapted from Cardinale et al., 2011).

Experimental evidence of the importance of the diversity-functioning relationship comes mainly from aboveground systems and aquatic habitats (e.g. Worm et al., 2006, Weis et al., 2008, Zavaleta et al., 2010, Cardinale et al., 2011). Most studies of terrestrial systems focused on plant productivity, not taking into account that plant production relies on the activity of the belowground system. However, some principles originally developed for the BEF relationship in plant ecology have been applied to microorganisms (e.g. Bell et al., 2005, Tiunov and Scheu, 2005, Jiang, 2007, Langenheder et al., 2010), in an effort to link microbial communities to ecosystem processes. Experimental tests with assembled bacterial communities at controlled richness levels have demonstrated that changes in species number can affect ecosystem processes. For instance, an early study investigating the effects of arbuscular mycorrhizal fungi (AMF) on plant diversity and functioning found that nutrient capture and plant productivity increased in macrocosms with increasing AMF-species richness inoculated from a pool of 23 different species isolated from environmental samples (van der Heijden et al., 1998). This represents perhaps one of the best examples of how microbial diversity can impact the functioning of a whole
ecosystem. Bell et al. (2005) investigated how diversity influences the functioning of bacterial communities through a microcosms experiment in which communities containing up to 72 species were constructed from a collection of culturable bacterial strains. Their results demonstrated that there is a relationship between community respiration and increasing bacterial diversity (Bell et al., 2005).

These early experiments opened the door to a series of studies manipulating the richness of microorganisms to unravel its relationship with community functioning. For instance, in manipulative liquid culture experiments, using all possible combination of six bacterial species, a positive linear relationship between species richness and metabolic activity has been observed, suggesting the involvement of both sampling and complementarity effects (Langenheder et al., 2010). However, in the same study, the shape of this relationship changed over time exhibiting saturation (Langenheder et al., 2010). There is also evidence of saturation at low species richness levels when redundant (i.e. functionally equivalent) species are present (Wohl et al., 2004), and negative sampling effects, where low performing species become dominant at high species richness levels (Jiang, 2007), or when the overall functioning is impaired by antagonistic interactions (Becker et al., 2012). On the other hand, the outcomes of experiments indirectly manipulating the diversity of microbial communities by means of dilution series (e.g. Langenheder et al., 2005, Wertz et al., 2006, Tardy et al., 2014), or fumigation (Griffiths et al., 2000) have failed to detect similar relationships. For example, Wertz et al. (2006) did not observe any relationship between communities of ammonia oxidisers, denitrifiers and heterotrophs with different richness and the associated functioning of these groups, i.e. nitrification, denitrification and carbon mineralisation.
This inconsistency in results might be due to key differences between the two approaches (Bell et al., 2009). In fact, they consider widely different richness levels, which is likely to alter the complexity of interactions. Additionally, fumigation or dilution series generate microbial communities that are subsets of the taxa originally present, whereas experiments with assembled communities can independently vary in richness and taxonomic composition. However, it can be argued that assembled communities are a too simplified representation of natural communities, and thus drawing firm conclusions from such experiments must be taken with caution.

Most studies on relationships between soil microorganisms and ecosystem function have been done in controlled microcosm experiments, but there is strong evidence from field studies that soil microbial communities are linked to ecosystem functioning (Hallin et al., 2009, Allison et al., 2013). In a long-term agricultural experiment with different fertilisation regimes genotype richness of denitrifying bacteria was found to affect ecosystem functioning under fluctuating conditions, which was interpreted as driven by enhanced complementarity due to increased genotypic dissimilarities (Hallin et al., 2012). Nonetheless, the number of studies investigating the relationship between microbial diversity and ecosystem functioning under natural conditions is limited, compared to artificial assemblages and indirect manipulations of diversity experiments. As a result, our understanding of the functional importance of species richness in the field is not complete, and it is not known how changes in soil microbial diversity across contrasting locations and land use systems impact ecosystem functioning.
1.2.1.2. Community Composition

There is ample evidence from aboveground systems that community composition and species identity may be as important as species richness in determining ecosystem functioning of different environments (Covich et al., 2004, Downing, 2005, Cardinale et al., 2006). It also became apparent that the consideration of functional traits is pivotal for the outcome of BEF research (Hillebrand and Matthiessen, 2009, Reiss et al., 2009). In fact, ecosystem response to changes in composition, due for instance to extinctions or invasions, will be determined both by the functional traits of species lost as well as the number of species that are lost.

As observed for plant communities, to some extent, not only the diversity level but also the composition of microorganisms matters to ecosystem functioning. In fact, the composition of microbial communities can influence the rates of biogeochemical processes and their response to changing environmental conditions (Schimel, 2001, Strickland et al., 2009). If whole functional groups, such as denitrifiers or methanogens, are lost or introduced, alteration of processes could occur at the ecosystem scale. Bell et al. (2005), through a manipulative experiment designed to separate the different contributions of species richness and species composition to bacterial functioning, found that differences in species composition are important in determining the level of community respiration. In another manipulative experiment, key functional traits among denitrifying bacteria were linked to the use of different carbon sources that strongly determined the functioning of assembled communities on a mix of carbon substrates (Salles et al., 2009, 2012). The complementarity of traits resulted as a much better predictor of denitrification than genetic richness
(Salles et al., 2012). These results support the hypothesis that the composition of bacterial communities plays a role in determining the level of ecosystem functioning. Contrasting results have been observed in aquatic environments, where specific enzyme activities varied in differently composed bacterial communities obtained with the dilution approach, whereas more universal bacterial functions such as biomass accrual and respiration remained similar because of the high number of similarly performing species (Langenheder et al., 2005, Peter et al., 2011a). This has been interpreted by the authors as a sign of weak coupling between composition of bacterial communities and their functioning, due mainly to functional redundancy. Results from field observations of natural soil diversity did not find any correlation between differences in composition of bacterial communities subjected to different agricultural treatments and differences in their corresponding potential activities (i.e. potential ammonia oxidation, denitrification, basal respiration and substrate-induced respiration) (Hallin et al., 2009). Correlations between functioning and the size of total bacteria and denitrifier communities were instead observed, suggesting a minor importance of community composition in predicting soil ecosystem functioning (Hallin et al., 2009).

Microbial community composition is often sensitive to disturbance and, thus, its shift in response to perturbation might be relevant to ecosystem processes. In fact, microbial composition is sensitive to extreme weather events, mineral fertilisation, C amendments and other disturbances (Allison and Martiny, 2008). Furthermore, in most of the cases microbial composition of disturbed communities usually differs from that of undisturbed ones, at least over the time scale of a few years (Allison and Martiny, 2008). In an environment where only a small percentage of the microbial population possesses the ability to provide a particular ecosystem service, species
composition should impact the rate of this narrow function if the group of species responsible for it is sensitive to perturbation. On the other side, large impacts might not affect functioning when a particular ecosystem function is carried out by a large portion of the microbial community. Therefore, changes in community composition due to disturbance can impact the rates of some ecosystem processes, suggesting that taxa in many microbial communities are not necessarily functionally redundant and different communities are not necessarily functionally similar. Changes of microbial composition have to be considered in order to understand the relations between microbial diversity and ecosystem functioning and further research is needed to make clear the actual role played by species composition in the regulation of microbial processes.

1.2.1.3. Evenness

Species evenness (or the complementary term, dominance) is one of the key components of biodiversity, together with species richness and species composition (Hooper et al., 2005). It is defined as the distribution of the number of individuals among species present in a given system and it reflects the distribution of traits in a community or in a functional group. Nowadays, the structure of communities is strongly altered by anthropogenic activities that affect biogeochemical cycles, abiotic factors, and trophic structure of the ecosystems. One of the effects that disturbance can have on natural communities is a change in the distribution of individuals among each species (Hillebrand et al., 2008). Whereas the effects of species loss on ecosystem functioning have received broad attention, especially in plant ecology (Hooper et al., 2012), the consequences of altered evenness on the processes carried out by ecosystems have been poorly investigated. Despite this, a change in evenness
can occur more rapidly than a change in species richness as a consequence of altered environmental constrains, and might also lead to rapid responses in ecosystem functions before species actually are driven to extinction (Chapin et al., 2000). In fact, if a community is dominated by one species and subjected to selective stress, this may lead to a great reduction of the abundance of that species, with a large impact on ecosystem functioning. This is less likely to happen in communities featured by a higher evenness as the proportion of individuals lost is likely to be smaller, and higher trait variability might allow communities to adapt to new environmental conditions. But considering a stable environment at a smaller temporal scale, any deviance from a certain successful trait by increasing evenness will reduce process rates. So, in this case, the highest level of ecosystem functioning will occur in a system highly dominated by the most active species and the divergence of resources into less productive species will reduce the overall functioning (Hillebrand et al., 2008). Thus, the effect of dominance comprises both the effect of the degree of dominance and the identity of the dominant species. Factors changing the dominance can alter the distribution of traits in a community, having consequences on the magnitude of intra- and interspecific interactions as well as community dynamics or processes depending on the traits distribution (Hillebrand et al., 2008).

The role played by evenness in the relationship between ecosystem functioning and biodiversity has been seldom taken into account in microbial ecology studies. Despite this, Wittebolle et al. (2009) showed in a manipulative experiment with denitrifying bacterial communities that evenness plays an important role in preserving the functional stability of a community. They found that the stability of
denitrification during a stress was strongly influenced by the evenness, concluding that in systems featured by high dominance the functioning of the more abundant species is less resistant to environmental stress. In a community where species are functionally redundant (that is, most contribute to the ecosystem function of interest), if initial evenness is high then the probability that a species tolerant to a perturbation is present is higher than when evenness is low. When evenness is low, meaning that the community is dominated by one or a few species, resistance to the perturbation will only occur if the dominant species are tolerant to the perturbation (Figure 1.2)

Doubtlessly, evenness is an important component of diversity that needs to be taken into account when investigating the BEF relationship. What is still lacking is a better understanding of its contribution to this relation in microbial systems and of the consequences that a change in species dominance can produce on the performance of the ecosystems.

**Figure 1.2** Relationship between the degree of evenness of a community and its stability when undergoing a perturbation (Adapted from Hillebrand *et al.*, 2008).
1.2.2. Impact of biodiversity loss on ecosystem functioning

Over the past decades, the effects of biodiversity loss and changes in the community structure and ecosystem processes have been the focus of much ecological research (e.g. Cardinale et al., 2012, Bardgett and van der Putten, 2014). The outcomes of this research provided evidences that global biodiversity loss has an impact on the functioning of the ecosystems (Cardinale et al., 2012). Several factors are involved in the decrease or change of ecosystem functioning. For instance, a reduction in the number of species and functional groups of primary producers reduces the efficiency of communities to uptake biologically essential resources and to convert them into biomass (Balvanera et al., 2006, Cardinale et al., 2011).

The shape of the BEF relationship in most experimental studies focusing on plants indicates that initial losses of species have relatively small impacts on ecosystem functions, but increasing losses cause accelerating rates of change (Cardinale et al., 2011). Therefore, the shape of the BEF relationship indicates the extent to which loss of the first species will influence ecosystem functioning (Reich et al., 2012). Consequently, the BEF relationship plays a paramount role in understanding and predicting the overall effects of perturbation on ecosystem functions. There are not yet quantitative estimates of the level of biodiversity loss at which changes in ecosystem functioning become significant. This represents an important area of research, whose development will predict the impacts of future changes in biodiversity on the ecosystems, and represents an important tool for efficient management and conservation policies.

In order to achieve this goal, research efforts must also be directed to microbial communities. In fact, contrary to what is generally thought, anthropogenic activities
affect not only larger organisms, but also have effects on microorganisms. It has been shown that microbial diversity in soil ecosystems is reduced by land-use intensification and increased nutrients inputs (Jangid et al., 2008, Culman et al., 2010), nitrogen deposition (Frey et al., 2004, Yao et al., 2014), and heavy metals contamination (Gans et al., 2005, Singh et al., 2014). High levels of stress can affect microbial communities, resulting in decreased microbial diversity and/or changes in community structure. Some niches become free and can be taken over by more resistant species that can perform better and thus increase their abundance, mainly due to lower competition and easier access to resources (Tobor-Kaplon et al., 2005). The loss of microbial biodiversity in soils represents an understudied field of research which requires more attention, especially for the conservation of the soil resource.

1.2.2.1. Biodiversity loss and functional groups: consequences for ecosystem functioning

A fundamental question is whether functional groups with a declining number of species can maintain process rates essential for ecosystem sustainability. Once there is sufficient functional redundancy, in fact, the gain and loss of species might not influence the overall rate of the processes catalysed. Schimel (1995) proposed that functional redundancy is greater for processes performed by a wide group of diverse microorganisms, such as respiration or decomposition, than for processes carried out by a narrow group of specific microorganisms. Some following studies supported this theory, suggesting that broad functions are unlikely to be affected by limited losses of diversity (Bardgett et al., 2008, Singh et al., 2010, Levine et al., 2011). An example of these broad functions is carbon mineralisation, which can be measured as
respiration. Carbon mineralisation involves a wide range of substrates metabolised by a large variety of microorganisms, therefore involving a major part of the total microbial community that likely possesses a high level of functional redundancy. A lack of impairment of soil respiration across diversity gradients has been reported across several experimental manipulations (Griffiths et al., 2001b, Wertz et al., 2006, Tardy et al., 2014). Despite this, some findings have challenged the functional redundancy theory, demonstrating that respiration and catabolic activity are influenced by diversity (Bell et al., 2005, Carney and Matson, 2005). Therefore, also widespread functions such as decomposition might be affected by a loss of microbial diversity. On the other hand, contrasting results have been found for narrow processes. For example, Wertz et al. (2006) demonstrated that reduced microbial diversity, did not significantly alter denitrification and nitrification processes, in contrast to other findings reporting significant effects of a reduction in denitrifier diversity on potential denitrification rates (Philippot et al., 2013). Also field observation did not report any link between estimates of total bacteria diversity and soil respiration, while the rate of methane consumption was found to increase with the diversity of methanotrophs (Levine et al., 2011).

More specialised functions, such as xenobiotic degradation, that are limited to one or a few species, might be even more sensitive to losses of diversity. For example, pesticide mineralisation, which is a function restricted to a few microbial taxa, has been shown to be compromised by a moderate loss of diversity, that did not impact broad functions such as basal and glucose-induced respiration (Singh et al., 2014).

Hence, microbial diversity is not necessarily functionally redundant, as often assumed, but it is likely to be functionally significant within more narrowly defined
functional groups. Furthermore, the relationship between functioning and diversity results in a more linear and less saturating response curve for specific functions. This plays a fundamental role in case of environmental perturbation, in which the performance of microbial communities may depend on the presence of rare as well as more abundant species. Further studies with explicit consideration of broad and specialised ecosystem functions in relation to soil microbial diversity are needed to further test these hypotheses, and to improve our limited knowledge of the extent of functional redundancy in microbial communities.

1.3. Ecological Sustainability

1.3.1. Defining ecosystem sustainability as ecosystem stability

Sustainability has been referred to the capacity for a given ecosystem service to persist at a given level for a long period of time (Lubchenco et al., 1991). Many authors dealing with sustainability relate it to the concept of ecosystem stability, which is defined by its two components resistance and resilience (Figure 1.3). Resistance is defined by the ability of a biological system to withstand a disturbance, while resilience is the rate at which the disturbed system is able to return to the original conditions (Pimm, 1984, Southwood, 1995). According to this definition, a system is sustainable when it is stable, i. e. when it is able to maintain its structure and functioning indefinitely (Costanza et al., 1992). Ecosystem functional stability refers to the ecosystem’s ability to minimize dynamic fluctuation and the ability to defy changes after disturbances (McCann, 2000). In soil ecosystem, resistance refers to the ability of soil functions to withstand a disturbance and resilience the ability to
recover to its non-disturbed level (Seybold et al., 1999). Both resistance and resilience are generally described as the ratio of functional parameters in perturbed treatments to those in unperturbed controls.

Resistance can be quantified through how little a measurement is changed by a given disturbance (Pimm, 1994), while resilience is measured as the proportional return toward pre-disturbance conditions (Tilman, 1996). Stability increases with the increase in resilience and resistance in the system (Griffiths et al., 2001a). High resilience does not mean low sensitivity to disturbance. It is a function of the type of disturbance (Fresco and Kroonenberg, 1992): the stronger the disturbance, the slower the recovery rate of the ecosystem will be. Longer return times after disturbance are an indication of loss of resilience (Ludwig et al., 1997).

Figure 1.3 Graph illustrating the effects of disturbance on resistance and resilience of ecosystem functioning (Adapted from Seybold et al., 1999).

From what is discussed above, it is evident that stability and, therefore sustainability, are not to be understood as the presence of static and unchanged conditions. Ecosystems are featured by a succession of several steady states that can differ in
structural complexity (Ludwig et al., 1997). They can cope with a certain amount of disturbance, but if this disturbance exceeds a threshold value the structure and functioning can change abruptly, and the ecosystem shift towards another steady state (Neumayer, 1998). Therefore, stability defines the limits of tolerable fluctuation without disrupting the system (Holling, 1973). However, the borderline between fluctuation and disturbance is difficult to assess.

1.3.2. Stability in microbial communities

Due to the important role that soil microbes play in driving plant productivity, abundance and diversity (van der Heijden et al., 1998, Schnitzer et al., 2011), the stability of their communities will have consequences for the functioning of the whole ecosystem. Despite the importance of microbiota, parameters related to microbial communities and processes are often excluded by the majority of ecosystem and climate models (Allison and Martiny, 2008), because changes in community composition and diversity are assumed not to affect ecosystem functioning. This assumption may be valid if the community is resistant, resilient and, therefore, stable. Microbial communities are usually considered by ecologists to be resistant and resilient. This is due to the high degree of metabolic flexibility and physiological tolerance to changing environmental conditions that some microbial groups show, and that could give stability to microbial communities. Also high abundances, widespread dispersal, the potential for rapid growth rates, and horizontal gene transfer make microbial communities resilient to disturbance (Fenchel and Finlay, 2004). Because of their fast growth rates, if their abundance is decreased by a disturbance event, microorganisms have the potential to recover quickly. Moreover, many microbes have a high degree of physiological flexibility so
that, even if the abundances of some taxa decrease initially, these taxa might physiologically adapt themselves to the new conditions and return to their original abundance (Finlay et al., 1997, Fenchel and Finlay, 2004). For instance, mixotrophy, or the ability to use many different carbon and energy sources, may be a common phenomenon in natural microbial communities (Eiler, 2006), providing individual flexibility in fluctuating environments. Dormancy is another physiological adaptation that has the potential to affect the stability of communities and ecosystem processes. Dormant individuals can be long-lived and contribute to seed banks, which can maintain species diversity (Chesson, 2000). This diversity may directly contribute to stability of microbial communities via niche complementation and/or functional redundancy. Seed banks may also aid in the recovery of microbial communities from severe disturbance events. For example, microbial communities show rapid recovery following whole ecosystem disturbances (Shade et al., 2012). Tolerance and adaptation of microorganisms to disturbance are important features for the overall stability of microbial communities to environmental change, and thus for the resistance and resilience of soil processes.

1.3.3. Biodiversity-stability relationship

One of the most controversial issues about the importance of biodiversity is the hypothesis that diversity influences stability. MacArthur (1955), Elton (1958) and Odum (1959) were among the first that hypothesised that greater diversity causes greater stability. Human simplification of natural environment represents a danger if ecosystems become less stable as they become simpler (Pimm, 1994). The consequence may be unstable populations leading to extinctions, further simplification and even more instability. However, contrasting responses in the
relationship between biodiversity and the stability of ecosystem processes have been reported, making it the centre of a long-standing debate in ecology (Loreau and Mazancourt, 2013, Gross et al., 2014). Species richness and evenness, have been shown to enhance the functional resilience of communities of larger organisms (Allison, 2004, Downing and Leibold, 2010, Van Ruijven and Berendse, 2010), whereas evidence about the impact of richness and evenness on microbial community stability is mixed (e.g. Griffiths et al., 2000, Wertz et al., 2007, Wittebolle et al., 2009).

The underlying mechanisms behind a positive relationship between diversity and stability has been suggested to be related to the ‘insurance hypothesis’ (Yachi and Loreau, 1999), according to which the high level of biodiversity confers an insurance against ecosystem malfunctioning under stress or disturbance. In fact, an ecosystem that retains its natural level of biological diversity will always contain some species that are more resistant to the new conditions, and that can thus keep the processes in the ecosystem going. The degree of functional redundancy can influence the stability and, therefore, sustainability of a microbial system. For instance, multiple populations may be able to perform a function, but they may not all perform it with the same efficiency or at the same time. So, if several species performing a given process are lost, the one that take their place in the system may not have the same ability or growth rate of the previous species, changing the overall functioning and stability. Therefore, a better understanding of the redundancy of microbial functions is required to make clearer the correlation between microbial diversity and the functions they carry out, in order to predict changes in ecosystem stability caused by diversity loss.
In an early study, Griffiths et al. (2000) used a series of fumigations followed by a disturbance to obtain different diversity levels and investigate the impact of diversity loss on functional stability. Communities with low diversity levels, were slower to recover after disturbance and, therefore, a lower resilience (measured as grass residue decomposition rate). Communities with higher diversity were, instead, more resistant and resilient to the applied perturbation. Since this early attempt to extend the diversity-stability research from plant community to soil microbial community, this kind of studies has been expanded, with different outcomes. A positive relationship between diversity and stability to copper sulphate and benzene amendments was found in soils with naturally differing levels of diversity (Girvan et al., 2005), while no correlation was observed in soils whose microbial diversity was reduced using serial dilutions (Wertz et al., 2007). In a recent study manipulating soil diversity by means of a dilution approach, functional stability improved with increasing diversity after heat disturbance, but not after mercury pollution, suggesting that the relevance of the insurance hypothesis might depend of the kind of disturbance (Tardy et al., 2014). These contrasting results could be due to the methods used to assess microbial diversity, the kind of disturbance applied, and the functions investigated. Our knowledge in this field is still limited, and more research is necessary to assess the importance of soil diversity in maintain soil stability. Understanding whether a threshold exists in the number of species necessary to maintain the ecosystem stability represents a challenge of paramount importance for scientific research, as it will help to predict the consequences of extinctions of species for the stability of ecosystem processes.
1.4. Aims of this study

The overall aim of the work described in this thesis was to investigate the relationship between bacterial communities and functioning and stability in soil ecosystems, in order to better understand the consequences of changes in microbial diversity on ecosystem functioning and sustainability. The purpose of this research was to provide direct evidence for the functional significance of microbial communities. Two interlinked objectives were addressed:

- To examine whether theoretical frameworks developed for plant diversity-ecosystem functioning relation can explain microbial regulation of soil functions;
- To determine the response of bacterial diversity to disturbance and the consequences for soil ecosystem sustainability.

Using a combination of microcosms experiments and field studies the following hypothesis were tested:

i. A decrease in bacterial diversity will have stronger effects on narrow functions, represented by lignin utilization, than on broad functions, community respiration and glucose utilisation;

ii. A decrease in bacterial diversity will have stronger effects on the stability to disturbance of narrow functions than of broad functions;

iii. The relationship between microbial communities and ecosystem processes involved in nutrient cycling will be influenced by environmental variables in natural systems;

iv. Resistance and resilience to disturbance of soil functions will be influenced
by bacterial diversity and community composition.
CHAPTER 2

Microbial diversity loss and functional stability to disturbance: dilution-to-extinction experiment
2.1. Introduction

The relationship between biodiversity and ecosystem functioning (BEF) has been the focus of recent ecological research, largely driven by the need to understand the consequences of ongoing loss of biodiversity on ecosystem processes and services (Hooper et al., 2012, Naeem et al., 2012). Most of the research on BEF has focused on plant ecology, supporting a positive relationship between diversity and ecosystem functioning (Cardinale et al., 2012). Other studies have evaluated the impact of biodiversity reductions on ecosystem stability following disturbance (e.g. Tilman, 1996, van der Heijden et al., 1998). Stability to disturbance is defined as resistance (i.e. the ability of the system to withstand disturbance) and resilience (i.e. the ability of the system to recover after disturbance (Pimm, 1984, Southwood, 1995).

Microbes are the most abundant organisms on Earth (Curtis and Sloan, 2005) and they are responsible for many key ecosystem functions, such as biogeochemical cycling, plant productivity and climate regulation (Bloem et al., 1997). Therefore, understanding how microorganisms influence ecosystem functioning and respond to disturbance is central to predicting the response of ecosystems to future environmental conditions in the context of global change. Despite this, our understanding of the role of microbial diversity in ecosystem functioning and stability is still incomplete. Some studies have addressed BEF in soil microbial communities but the results are contrasting, depending mainly on the function considered (e.g. Griffiths et al., 2001b, 2004, Wertz et al., 2006, Philippot et al., 2013). For example, when focusing on general functions, such as soil respiration or litter decomposition (Degens, 1998, Griffiths et al., 2000, 2001b, Müller et al., 2002), no relationship between microbial diversity and functional stability was
found. Because most microorganisms in soil are heterotrophs and are potentially decomposers of organic matter, functional redundancy is expected to be high, therefore loss of diversity may not impact this function. On the other hand, studies that link diversity with more specialised functions, such as denitrification and nitrite oxidation, and their stability reported both negative or no effects of a decrease in diversity on functioning (Wertz et al., 2007, Philippot et al., 2013).

The aim of this study was, therefore, to investigate the role of diversity on the functioning of soil microbial communities and the impact of a loss of diversity on their functional stability following a model disturbance (i.e. heating). Community respiration and the ability to degrade compounds of different complexity, such as glucose and lignin, were chosen as functional parameters. Glucose is a labile compound that can be utilised by the vast majority of microbes, thus representing a widespread function, while lignin is recalcitrant and is mineralised by only a few groups of microorganisms in soils (Goldfarb et al., 2011). It was hypothesised that a loss of biodiversity will have a stronger effect on the stability of narrow functions such as the degradation of lignin than of broad functions, here represented by the utilisation of glucose. Soil diversity was manipulated by using the dilution-to-extinction approach that preferentially removes rare species (Salonius, 1981). According to this method, less abundant species are removed first, creating mixtures of organisms differing in diversity. Regrowth of the diluted mixtures should then produce cultures of approximately the same biomass, but differing in overall diversity. This method has been widely used in studies manipulating the microbial diversity in several environments, for instance sewage water (e.g. Franklin et al., 2001, Franklin and Mills, 2006), freshwater (Peter et al., 2011b, Szabó et al., 2007),
and soil (e. g. Griffiths et al., 2001b, 2004, Wertz et al., 2006, 2007, Philippot et al., 2013).

In the present study the microbial community was extracted from a soil sample and inoculated in soil extract medium (SEM), made from the soil where the inoculum was extracted from. The dilution-to-extinction approach was used to create a diversity gradient, which was confirmed by a DNA fingerprinting method. Stability to disturbance of the diluted community was investigated by assessing both resistance and resilience of respiration and degradation of lignin and glucose to heat disturbance. By growing soil microorganisms in liquid medium that reproduces the chemical properties of the original soil, I removed the potential influence of the soil structure and associated variables, which can affect the expression of functions in soil system.

2.2. Materials and methods

2.2.1. Soil sampling and soil characteristics

The soil used in this experiment was collected from the 0-20 cm layer of pasture grassland adjacent to the Hawkesbury Forest Experiment site (33°36’40”S, 150°44’26.5”E), University of Western Sydney, Richmond, NSW, Australia. The soil was a sandy loam with low organic matter content (0.7%), low fertility (available P, 8 mg kg⁻¹; exchangeable cations K 0.19 mEq 100 g⁻¹; Ca 1.0 mEq 100 g⁻¹; Mg 0.28 mEq 100 g⁻¹) and low water holding capacity (Barton et al., 2010), with a pH of 5.85.
The amount of soil necessary for the preparation of culture media and for the inoculum was collected and immediately stored in plastic bags at 4 °C in the dark until analyses.

2.2.2. **Preparation of soil extract media (SEM)**

Fresh soil was sieved through a 2 mm mesh to remove stones, roots, and larger invertebrates. A portion of the soil was used for the inoculum, while the remaining part was autoclaved with twice its volume of distilled water (*e.g.* 1 kg soil in 2 litres) for 16 minutes at 121 °C. The mixture was centrifuged at 3200 x g for 10 minutes (Centrifuge 5810R, Eppendorf) to remove large soil particles in 50-ml tubes. After autoclaving the liquid a second time as above, the pH was adjusted to the value of the soil before treatment by adding filter-sterilised 1M NaOH or 1M HCl. The pH was measured with a Delta pH-meter (Mettler-Toledo Instruments, Columbus, OH, USA).

2.2.3. **Extraction and dilution of soil microbial community**

The microbial community was extracted by mixing soil in SEM with a ratio of 1 g: 10 ml with 10 glass beads (5 mm diameter, Sigma Aldrich, Australia) in 50 ml centrifuge tubes. The suspension was shaken vigorously for 20 seconds and vortexed for 2 minutes. The tubes were then centrifuged at low speed (500 x g) for 5 minutes (Centrifuge 5810R, Eppendorf). The supernatant was transferred into three sterile 250-ml Erlenmeyer flasks resulting in an initial total volume of 200 ml each. These microcosms represented the undiluted controls. Ten-fold serial dilutions were then performed from each of these undiluted controls in 200 ml sterile SEM until a dilution of $10^{-8}$ was reached. The $10^{-2}$, $10^{-4}$, $10^{-6}$, and $10^{-8}$ dilutions were used for
further studies. Three flasks containing 200 ml of sterile SEM were added to the experiment as controls. Half of the SEM and microbial community suspension contained in each flask was transferred to another autoclaved flask, obtaining two sets of microcosms: one set was incubated at 25 °C for the whole duration of the experiment (eight days), representing the control, and the other set of microcosms was shocked at 50 °C for 30 minutes, after biomass recovery, and then incubated again at 25 °C. The temperature and duration of the heat treatment were chosen after running a trial experiment (see Appendix A). All the steps were carried out under sterile conditions.

A total of 42 liquid microcosms were created following the above procedure: 5 dilutions, 1 undiluted control, and 1 sterile control, all of them replicated 3 times, in two sets (Figure 2.1).

![Figure 2.1](image-url) Schematic representation of the design used to test the relationship between biodiversity, function and stability.
2.2.4. Biomass recovery monitoring

The liquid microcosms were pre-incubated on a horizontal shaking incubator (OM15, Ratek, Australia) for four days at 25 °C at 150 r.p.m., to allow for biomass recovery after the dilution treatment. Biomass was considered fully recovered when cell abundances similar to the undiluted control were observed in all the dilution treatments. The pre-incubation time was chosen after carrying out trial experiments that showed that microbial biomass always reached the level observed in the undiluted control after four days (see Appendix B). The sterility of the media was checked by plating 100 μl aliquots from each of the sterile control microcosms on triplicated nutrient agar plates (LB agar, Sigma Aldrich, Australia), and monitoring growth during incubation at 25 °C in the dark over three days. Sterility was confirmed when no growth was observed over the three days period.

The biomass recovery was assessed by means of bacterial quantitative polymerase chain reaction (qPCR) targeting the 16S rRNA gene. Aliquots of culture of 1 ml were sampled and bacterial pellets collected following centrifugation at 21130 x g for 1 minute for the quantification of bacterial abundances immediately after dilution and after again four days of incubation. DNA was extracted by using the PowerSoil® DNA Isolation kit (MoBio Laboratories, CA, USA). The pellets containing bacterial cells were resuspended in 50 μl of sterile deionised water and loaded into a single Power Soil® Bead tube. DNA was extracted following the manufacturer’s specifications from the cell-lysis step, with modifications that a FastPrep bead beating system (Bio-101, Vista, CA, USA) at a speed of 5.5 m s⁻¹ for 30 seconds was used. Once DNA was extracted, 16S rRNA qPCR was carried out for the quantification of total bacteria, as detailed below.
qPCR assays were conducted on a Rotor-Gene 6000 cycler (Qiagen, Australia). Each 25 μl reaction contained 12.5 μl of GoTaq® qPCR Master mix (Promega, Australia), 0.63 μl of Eub 518R and Eub 338F primers (20 pmol/μl; Sigma Aldrich, Australia, sequences listed in Table 2.1), 2 μl bovine serum albumin (BSA, 20 mg/ml; New England Biolabs, USA), 4.24 µl nuclease-free water (Ambion, Life Technologies, Australia) and 5 μl template DNA. PCR conditions were 5 minutes at 95 °C, followed by 30 cycles of 94 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 1 minute, and 83 °C for 15 seconds. The last cycle was followed by 10 minutes at 72 °C. Each run included the appropriate set of standards in duplicate and two negative control samples (without DNA).

Table 2.1 Oligonucleotides used for the amplification of bacterial ribosomal DNA in qPCR reactions.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Target region and specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eub338F</td>
<td>ACTCCTACGGGAGGCAGC</td>
<td>16S rRNA gene</td>
<td>(Amann et al., 1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eubacteria</td>
<td></td>
</tr>
<tr>
<td>Eub518R</td>
<td>ATTACCGCGGCTGCTGG</td>
<td>16S rRNA gene</td>
<td>(Muyzer et al., 1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eubacteria</td>
<td></td>
</tr>
</tbody>
</table>

To produce a standard curve, a plasmid standard containing the target region was generated using DNA extracted from a positive control strain. Amplified products were run on 2 % agarose gel to confirm product size and specificity, and cloned using the TOPO TA cloning kit (Life Technologies, Australia). Plasmids were isolated using the Qiaprep Plasmid Miniprep kit (Qiagen, Australia) with DNA concentrations determined using a Qubit 2.0 fluorometer (Life Technologies, Australia). Standard curves were generated in duplicate via 10-fold dilutions of the quantified PCR amplicon. Five standard concentrations per assay were included,
with standard concentration ranging from $10^9$ to $10^5$ copies $\mu l^{-1}$. Melt curve analysis of the PCR products was conducted after each assay (ramp from 65 to 94 °C, rising by 1 °C each step) to confirm that the fluorescence signal originated from specific PCR products and not from primer dimers or other artefacts (RotorGene Q Series Software 2.2.3, Qiagen, Australia).

### 2.2.5. Determination of relative bacterial diversity and richness

After biomass had recovered, DNA was extracted as described above and terminal restriction fragment length polymorphism (T-RFLP) analysis was performed. DNA samples were amplified by PCR targeting 16S rRNA genes, using 63F-VIC and 1087R-FAM primers labelled at the 5’ end with VIC and FAM fluorescent dyes respectively, specific for bacteria. The sequences of the primers used are listed in Table 2.2 with relative label, sequence, target region and specificity.

**Table 2.2** Oligonucleotides used for the amplification of bacterial ribosomal DNA in PCR reactions for T-RFLP.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Fluorescent label</th>
<th>Sequence (5’ to 3’)</th>
<th>Target region and specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>63F</td>
<td>VIC</td>
<td>AGGCCTAACACATGCAAGTC</td>
<td>16S rRNA gene Eubacteria</td>
<td>(Marchesi et al., 1998)</td>
</tr>
<tr>
<td>1087R</td>
<td>FAM</td>
<td>CTCGTTGCGGGACTTACCC</td>
<td>16S rRNA gene Eubacteria</td>
<td>(Hauben et al., 1997)</td>
</tr>
</tbody>
</table>

### 2.2.5.1. PCR amplification

The PCR master mixture contained, for a total reaction volume of 50 $\mu l$, 5 $\mu l$ PCR Reaction Buffer (10x, Bioline, Australia), 1.5 $\mu l$ MgCl$_2$ at a concentration of 50 mM, 1 $\mu l$ deoxynucleoside triphosphate (dNTPs, Bioline, Australia) at a concentration of
10 mM, 20 pmol of each primer (Sigma Aldrich, Australia), 0.5 µl of BSA (20 mg/ml, NewEngland BioLabs, USA), 1 unit BIOTAQ DNA polymerase (Bioline, Australia), and 1 µl of DNA extraction as the template. The program consisted of an initial step of 5 minutes at 95 °C, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, and elongation at 72 °C for 1 minute. The last cycle was followed by extension at 72 °C for 5 minutes. The PCRs were carried out in a Dyad Peltier thermal cycler (BioRad, Australia).

PCR amplicons were stained with 0.1 µl/ml SybrSAFE (10.000x, Life Technologies, Australia) and visualised on a 1% (w/v) agarose gel using UV radiation. PCR products were purified using the Charge Switch® PCR cleanup kit (Life Technologies, Australia) following the manufacturer’s instructions. Purified PCR product concentrations and purity were determined with a NanoDrop™ ND-2000c UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA concentration was then used to normalise the amount of DNA at the restriction digestion step.

2.2.5.2. Digestion of PCR products and fragment analysis

All PCR products were digested with 1 µl HhaI restriction endonuclease (20,000 x, NewEngland BioLabs, USA) in 20-µl reaction mixtures containing 200 ng of PCR products, 2 µl of Buffer #4 (10 x, NewEngland BioLabs, USA), and 0.2 µl of BSA (20 mg/ml, NewEngland BioLabs, USA). Samples were incubated at 37 °C for 3 hours, followed by a deactivation step at 65 °C for 20 minutes.

DNA fragment analysis was carried out on an ABI PRISM® 3130xl Genetic Analyser following manufacturer’s instructions (Applied Biosystems, Life Technologies, Australia). The analyser was equipped with an 8-channel 50-cm
capillary system which was loaded with POP-7 polymer and calibrated with a DS-33/G5 Matrix Standard Kit. The sizing standard GeneScan 600 LIZ v2.0 was used in each sample. The size and quantity of terminal restriction fragments were analysed using GeneMapper software (version 4.1, Applied Biosystems, Life Technologies, Australia). Terminal fragments with a length less than 80 base pairs (bp) were excluded from the analysis to avoid noise. Baseline was set up based on overall fluorescence noise of each run to exclude peaks resulting from technical artefacts.

Resulting data were imported into T-REX software (http://trex.biohpc.org, Culman et al., 2009) for further processing. Data were subjected to quality control procedures including terminal restriction fragment (TRF) alignment (clustering threshold = 0.9 bp), and noise filtering (peak area, standard deviation multiplier = 1). Relative abundance tables were generated based on peak height.

2.2.5.3. **Diversity indices**

Relative abundance tables were used to extrapolate measures of bacterial diversity and richness. Diversity was measured by the Simpson’s diversity index (Equation 2.1; Simpson’s, 1949), and the evenness by the Pielou’s index (Equation 2.2; Pielou, 1966)

**Equation 2.1** Simpson’s diversity index. \( n_i = \) relative abundance of the \( i^{th} \) terminal restriction fragment (TRF); \( N=\)total number of TRFs.

\[
\sum_{i=1}^{S} \frac{n_i(n_i - 1)}{N(N - 1)}
\]
Equation 2.2 Pielou’s index of evenness \( (J') \). \( S \) = total number of TRFs; \( p_i \) = relative abundance of each TRF. \( H' \) represents the Shannon diversity index.

\[
J' = \frac{H'}{\ln S} \quad \text{and} \quad H' = -\sum_{i=1}^{N} p_i \ln p_i
\]

Richness was measured as the total number of TRFs. The mean of Simpson’s diversity indices deriving from forward and reverse TRF analysis was used as an overall relative diversity estimate. The same procedure was followed for richness and evenness indices.

2.2.6. Functional measurements

Once the biomass had recovered to statistically similar values, community respiration rates were assessed using the MicroResp™ method (Campbell et al., 2003). Respired CO\(_2\) results in a change in colour of cresol red indicator dye suspended above each well of a 96-well microplate. For each sample, four replicates of 600 µl were used per dilution in a single plate. Three separate 96-deep well microplates were used. One plate was amended with D-(+)-Glucose, another with lignin (both obtained from Sigma Aldrich, Australia) reaching the final concentration of 1 mg ml\(^{-1}\) per well. The third plate was used for measuring basal community respiration, therefore it was not amended. The optical density of the CO\(_2\) detection plates was measured immediately before the deep-well plates were sealed on a spectrophotometer microplate reader (EnSpire\textsuperscript{®} 2300 Multilabel Reader, Perkin Elmer, USA) set to record a colour development at a wavelength of 570 nm. Immediately after the addition of carbon sources the deep-well plates were sealed with CO\(_2\) detection plates and incubated on a shaking incubator at 25 °C for 6 hours as recommended by the manufacturer (Macaulay Scientific Consulting, UK). The
change in optical density of the CO\textsubscript{2} detection plates was then measured again after the incubation. The rate of CO\textsubscript{2} respiration per millilitre of culture medium was calculated using the following formula derived from the MicroResp\textsuperscript{TM} manual (Macaulay Scientific Consulting, UK):

\[
\text{CO}_2 \text{respiration} = \frac{\left(\% \frac{\text{CO}_2}{100}\right) \times \text{headspace volume} x \left(\frac{44}{22.4}\right) x \left(\frac{12}{44}\right) x \left(\frac{273}{273 + 25^\circ \text{C}}\right)}{\text{ml} \times \text{incubation time}}
\]

2.2.7. Disturbance and measurement of stability

Stability (resistance and resilience) was measured as the response of the soil microbial community to a heat treatment compared to undisturbed control microcosms. The microcosms were shocked for 30 minutes at 50 °C and the respiration rates were monitored as described in section 2.2.6 immediately after the shock and then four days following shock.

2.2.7.1. Resistance

Resistance was calculated by means of a resistance index (Orwin and Wardle, 2004) separately for each of the three functions (i.e. basal respiration, glucose-induced respiration and lignin-induced respiration). This resistance index (Equation 2.3) is bounded by -1 and +1, with +1 representing full resistance (the disturbance does not produce any change), an index value of 0 indicating either a 100% increase or decrease following disturbance, and -1 representing the lowest resistance and, therefore the largest change after disturbance.

\textbf{Equation 2.3 Index of resistance}

\[
\text{Resistance (RS)} = 1 - \frac{2 \mid \text{Control} - \text{Disturbed} \mid}{(\text{Control} + \mid \text{Control} - \text{Disturbed} \mid)}
\]
This index was developed for soil functions and only deals with absolute differences between control (i.e. non disturbed) and disturbed values, and is standardised by the control value to allow comparison between different soils. It has previously been shown to give an accurate quantification of resistance (Orwin and Wardle, 2004).

2.2.7.2. Resilience

Resilience was calculated by means of a resilience index as described by Orwin and Wardle (2004) for each of the three functions, based on the respiration rates measured four days after disturbance in the control and the perturbed microcosms. This index of resilience (Equation 2.4) is also bounded by -1 and +1, where the latter represents the full recovery to pre-shock conditions and, hence, maximum resilience, and lower values indicate a slower recovery. So if the absolute value of the difference between the control and the disturbed microcosms at the time point chosen is between 0 and the absolute value of the difference measured after the disturbance was applied, the index will give values between 0 and 1. When the difference between control and disturbed microcosms at time 0 is higher than the difference at the time point chosen, the index will have a negative value, indicating no recovery. An index value of 0 indicates either no recovery after the disturbance, or that the disturbed treatment is different to the control by the same amount, but in the opposite direction. This index of resilience has previously been shown to give an accurate representation of resilience (Orwin and Wardle, 2004).

**Equation 2.4** Index of resilience. $t_0$ represents the time in which the shock is applied, and $t_4$ is the time point chosen to measure resilience.

\[
\text{Resilience (RL)} = \frac{2|\text{Control} - \text{Disturbed}|_{t_0}}{|\text{Control} - \text{Disturbed}|_{t_0} + |\text{Control} - \text{Disturbed}|_{t_4}} - 1
\]
2.2.8. Statistical methods

All the differences between dilution treatments were assessed by means of a one-way analysis of variance (ANOVA). Where significant differences for the main effect were observed ($P < 0.05$), a Tukey’s pairwise comparison test was also performed. Before carrying out ANOVA, dependent variables were tested for normality and homoscedasticity using the Shapiro-Wilk’s test and the Bartlett’s test, respectively. 16S rRNA copies numbers were log-transformed in order to meet the normality assumptions of the ANOVA. The relationship between bacterial diversity and richness and functioning was analysed by means of univariate linear regressions. All the analyses were carried out by using the R software version 3.1.1 (R Core Team 2014).

2.3. Results

2.3.1. Bacterial biomass recovery

Before testing the hypotheses related to the diversity-functioning relationship, biomass of the microcosms was monitored to assess its recovery after the dilution treatment. The 16S rRNA gene copy number per millilitre of culture was $7.12 \times 10^5$ before dilutions were performed, while in the diluted cultures and in the sterile controls, it ranged from $1.27 \times 10^4$ ($10^{-6}$ dilution) to $1.66 \times 10^4$ ($10^{-2}$ dilution), as measured on the same day of the inoculum (T0) (Figure 2.2). Since no growth was observed on LB agar plates, the bacterial abundance estimated in the sterile controls was due to the presence of DNA from non-living cells in the culture medium.
Figure 2.2 Bacterial biomass recovery at different dilution levels: data of copies number ml$^{-1}$ of bacterial 16S rRNA gene. Different letters within a time point indicate abundances that are statistically different (T0: $P < 0.001$; T5: $P < 0.01$). Error bars indicate ± one standard error (n=3).

A significant difference between the undiluted treatment and all the dilutions and sterile control was observed at T0 ($F_{5,12} = 65.97, P < 0.001$), indicating that the dilution approach effectively decreased the bacterial abundance.

After five days of pre-incubation, quantification of 16S rRNA gene showed globally similar abundances, independent of dilution level (Figure 2.2).

All the treatment showed values ranging from $1.8 \times 10^7$ (undiluted treatment) to $5.46 \times 10^7$ ($10^{-8}$ dilution) copies ml$^{-1}$. The copy number of the sterile control were statistically different from the rest of the treatments ($F_{5,12} = 7.52, P = 0.002$) at the end of the pre-incubation time (T5), indicating that no growth took place over this time. The dilution treatment therefore had no significant effect on the abundance of bacterial cells after five days recovery.
2.3.2. Effects of dilution on bacterial diversity

Once biomass recovery was achieved, relative bacterial diversity was estimated through T-RFLP analysis. The relative abundances of OTUs were used to estimate diversity, richness, and evenness of the bacterial communities. The dilution approach significantly affected OTU richness (Figure 2.3A) \((F_{4, 9} = 4.32, P = 0.031)\), with an overall reduction of 77% in the number of OTUs (comparison of \(10^{-2}\) to \(10^{-8}\)). The average OTU richness ranged from 6.33 ± 1.42 in the undiluted treatment, with an apparent increase in the \(10^{-2}\) dilution (10.75 ± 1.25) and a linear decrease thereafter to 2.5 ± 1.04 in the highest dilution (\(10^{-8}\)). The diversity index (Figure 2.B) followed a similar trend to the richness index, with values of the Simpson’s index ranging from 0.69 ± 0.14 (undiluted treatment) to 0.32 ± 0.16 (\(10^{-8}\) dilution), but differences across dilutions were not significant \((F_{4, 9} = 2, P = 0.177)\). Pielou’s evenness index (Figure 2.3C) showed similarly high values in the undiluted control (1x), \(10^{-2}\), \(10^{-4}\), and \(10^{-6}\) dilution (ranging between 0.77 ± 0.15 to 0.85 ± 0.04), decreasing only in the \(10^{-8}\) dilution (0.48 ± 0.22). The evenness index showed no significant differences between treatments \((F_{4, 9} = 1.10, P = 0.410)\).
Figure 2.3 Effects of dilution on relative OTU richness (A), diversity (B), and evenness (C) based on T-RFLP data. Values with different letters differ significantly (P < 0.05). Error bars indicate ± one standard error (n=3).
2.3.3. Effects of dilution on function

Once biomass recovery was achieved, an experiment to test the relationship between diversity and function was initiated. Two functions were tested: community respiration and utilization of glucose and lignin. Respiration data provide overall information on the total communities’ activity at the different levels of diversity, while the degradation of glucose and lignin is representative of broad and specific functions, respectively (Figure 2.4).

The diversity gradient produced by the dilution-to-extinction approach significantly affected the basal respiration ($F_{4, 10} = 4.07, P = 0.032$) of microbial communities (Figure 2.4A). Overall reduction in respiration (comparison of $10^{-2}$ to $10^{-8}$) was 41%.

The respiration in presence of glucose amendment was not observed to be affected by the dilution of the microbial community ($F_{4, 10} = 2.91, P = 0.077$, Figure 2.4B).

The degradation of lignin was significantly affected by the dilution treatment ($F_{4, 10} = 3.68, P = 0.042$, Figure 2.4C). Overall reduction in respiration (comparison of $10^{-2}$ to $10^{-8}$) was 33%.
Figure 2.4 Respiration rates measured after biomass recovery along the dilution gradient for: A) control (i.e., not amended), B) glucose, and C) lignin. Values with different letters differ significantly ($P < 0.05$). Error bars indicate ± one standard error (n=3).
2.3.4. Bacterial diversity and richness: relationship with functioning

The link between diversity and function was visualised by plotting the relative bacterial diversity and richness, estimated via T-RFLP analysis, against respiration rates measured in the control and after glucose and lignin amendments, as proxies of community functions (Figure 2.5 and Figure 2.6).

A positive linear correlation was observed between diversity and basal respiration (Figure 2.5A). However, the relationship was not confirmed when analysed in a univariate regression analysis ($F_{1, 12}= 0.97$, $P = 0.342$). In presence of glucose amendments, no relationship was observed between diversity and respiration ($F_{1, 12}= 0.006$, $P = 0.937$, Figure 2.5B). A positive relationship was observed between diversity and lignin degradation (Figure 2.5C). However, also in this case the relationship was not confirmed when analysed in a univariate regression analysis ($F_{1, 12}= 0.92$, $P = 0.355$).

On the other hand, bacterial richness had stronger effects on basal respiration ($F_{1, 12}= 3.96$, $P = 0.069$, Figure 2.6A) and on the degradation of lignin ($F_{1, 12}= 4.66$, $P = 0.051$, Figure 2.6C), but no effect was observed for the utilisation of glucose ($F_{1, 12}= 0.006$, $P = 0.937$, Figure 2.6B) when analysed in a univariate regression analysis.
Figure 2.5 Relationship between bacterial diversity and respiration. Bacterial diversity is estimated by T-RFLP analysis. A) control, B) samples incubated with glucose, C) samples incubated with lignin.
Figure 2.6 Relationship between bacterial OTU richness and respiration. Bacterial diversity is estimated by T-RFLP analysis. A) control, B) samples incubated with glucose, C) samples incubated with lignin.
2.3.5. Resistance to disturbance along a diversity gradient

The values of the resistance index were high (Figure 2.7), indicating that the heat shock did not produce a large change in the communities’ respiration rates. The non-diluted microcosms had average resistance index of 0.79 ± 0.11, which increased to 0.92 ± 0.03 in the 10⁻² dilution, decreasing again in the following dilution to 0.38 ± 0.12 in the 10⁻⁶ treatment. The highest dilution (10⁻⁸) showed a slight increase of the resistance index compared to the previous dilution (i.e. 0.58 ± 0.15). The overall reduction in the resistance index between the undiluted treatment and 10⁻⁸ dilution calculated on basal respiration data was 30%. Overall, the dilution-to-extinction approach affected significantly (F₄,₁₀ = 3.85, P = 0.038) the resistance to heat shock of the community respiration (Figure 2.7A), whereas no significant differences were observed with glucose (F₄,₁₀ = 0.22, P = 0.919, Figure 2.7B) and lignin (F₄,₁₀ = 1.38, P = 0.307, Figure 2.7C) amendments.
Figure 2.7 Resistance indices calculated on respiration rates data collected before and after shock along the dilution gradient for A) control, B) glucose, and C) lignin. Values with different letters differ significantly ($P < 0.05$). Error bars indicate ± one standard error (n=3).
2.3.6. Resilience to disturbance along a diversity gradient

The resilience index had low values for all the dilution treatments (Figure 2.8), indicating that none of the communities fully recovered their functionality four days after disturbance to the control’s levels. Generally, the index values were lower for the diluted communities than for the undiluted controls, for all the measured functions, although these differences were not significant ($P > 0.05$). The resilience index calculated on basal respiration data had the highest value in the undiluted treatment ($0.01 \pm 0.41$) and the lowest in the $10^{-2}$ dilution ($-0.57 \pm 0.16$). The rest of the treatments had resilience index values ranging from -0.39 to -0.56 (Figure 2.8A).

For the glucose utilisation, the highest value of the resilience index was recorded in the undiluted treatment ($-0.049 \pm 0.21$) and the lowest in the $10^{-6}$ dilution ($-0.62 \pm 0.12$). The rest of the treatments had resilience index values ranging from -0.22 to -0.48 (Figure 2.8B). The undiluted treatment showed the highest value of the index for the degradation of lignin ($-0.09 \pm 0.32$), while the rest of the treatments had values ranging from -0.40 to -0.61, with the latter being lowest value recorded in the $10^{-8}$ dilution (Figure 2.8C).
Figure 2.8 Resilience indices calculated on respiration data collected before shock, after shock, and four days after shock along the dilution gradient for A) control, B) glucose, and C) lignin. Error bars indicate ± one standard error (n=3).
2.4. Discussion

2.4.1. Dilution-to-extinction approach, biomass recovery and bacterial diversity

The aim of this study was to evaluate the impact of a reduction in microbial diversity on the functioning and stability of soil microbial communities in liquid culture. Fungi play an important role in soil ecosystem functions including community respiration (Dighton, 2003, Six et al., 2006) but, because they are known not to develop significant growth in liquid media, which was confirmed by the failure in amplifying fungal genes by PCR during this experiment (data not shown), this study did not investigate fungal diversity. A bacterial diversity gradient was obtained via a dilution-to-extinction approach (Salonius, 1981), carried out in soil extract medium. As reported by Gans et al. (2005), the removal of less abundant species represents a realistic scenario of microbial diversity decline following anthropogenic disturbance. T-RFLP is well known to underestimate species richness because populations that are not numerically dominant are not represented if their template DNA comprises too small a fraction of the total community DNA (Dunbar et al., 2000). Moreover, due to the conservation of restriction site positions in the 16S rRNA gene, the resolution of T-RFLP analysis is not at the species level but reflects the distribution of higher-order groups (Franklin et al., 2001). Despite these limitations, T-RFLP has been demonstrated to effectively estimate differences in bacterial diversity and richness along a dilution gradient, being an appropriate reflection of the more informative data provided by pyrosequencing (Giaramida, 2013) for freshwater systems. Based on our T-RFLP results, the dilution-to-extinction approach was an effective method to manipulate richness of soil bacterial communities cultured in
SEM, confirming previous observations in soil microcosms experiments (Griffiths et al., 2001b, Wertz et al., 2006). However, some reports showed contrasting results in liquid culture experiments, where dilution treatment did not follow a significant reduction in diversity/richness when measured via T-RFLP and amplified fragment length polymorphism (Franklin et al., 2001, Franklin and Mills, 2006). In our study, a six-orders-of-magnitude dilution (i.e. comparison of $10^{-2}$ to $10^{-8}$) produced a consistent reduction in diversity and richness of 60% and 77%, respectively.

2.4.2. Relationship between bacterial diversity and community functioning

Several experiments used the removal approach (i.e. fumigation or dilution) for reducing microbial diversity in microcosm experiments, with varying results for the relationship between diversity and functioning. A detrimental effect of dilution treatment on freshwater biofilm functioning was reported by Peter et al. (2011), where they observed a decline in chitin and cellulose degradation rates along the dilution gradient. Franklin et al. (2001) reported that dilution treatment reduces the number of carbon sources that sewage water microbial communities are able to metabolise, while Franklin and Mills (2006) did not find a significant effect of a dilution gradient on the metabolism of glucose, acetate, citrate and an amino acid mixture. Griffiths et al. (2001b) did not find any decrease in a wide range of soil processes in soil microbial communities with reduced diversity achieved by dilution. Similar results were found when considering the reduced diversity of specific functional groups (Wertz et al., 2006) with no impairment of the associated functioning of ammonia oxidizers, denitrifiers, and heterotrophs (i.e. nitrification, denitrification, and carbon mineralisation). On the other hand, Philippot et al. (2013)
observed significantly lower potential denitrification activity in soil microcosms with decreasing diversity.

These divergent observations could depend on the function measured, experimental approach, environmental properties, and a combination of these factors. Also differences in the original microbial communities across aquatic environments and across soils may play an important role in producing these contrasting results. In fact, communities differing in their structure and composition may show different patterns of ecosystem response to diversity loss with some being more sensitive than others (Philippot et al., 2013). In this study, the reduction in bacterial richness of 77% (comparison of $10^{-2}$ to $10^{-8}$) led to a significative decrease in respiration rates of 41% in the control (i.e. no addition of carbon sources) and of 33% with addition of lignin, but did not affect the glucose-induced respiration rate. Moreover, a positive linear correlation, even if marginally non significant, was found between OTU richness and community respiration and lignin-induced respiration ($P = 0.06$ and 0.05 respectively), but it was not possible to identify a clear relationship with the utilisation of glucose. These results provide the evidence that diversity loss can affect differently the functioning of microbial communities. Specific functions such as the utilisation of lignin, a recalcitrant compound that can be broken down by a limited subset of bacteria (Vicuña, 1988, Zimmermann, 1990), showed a stronger positive relationship with species richness, compared to overall respiration and the utilisation of glucose. Therefore specific functions may be significantly more affected by a reduction of diversity than general functions. Moreover, it is possible to observe that the slopes of the correlation between diversity and richness with lignin utilisation were steeper than those of diversity and richness with community respiration. This indicates that the relationship between biodiversity and generic
functions, such as respiration, has a reduced slope, and thus strength, because of the reduced contribution of additional species to the ecosystem functioning. CO₂ production, as a measure of overall microbial activity, results from many distinct metabolic processes catalysed by different microorganisms. Since none of these necessarily limits the rate of CO₂ production, respiration represents an aggregate ‘broad’ function, and therefore changes in microbial diversity are expected to have less impact on respiration (Schimel, 1995). Thus, any additional species increases niche overlap thus producing a nonlinear, saturating BEF relationship (Gravel et al., 2011), as hypothesised in this study. These findings are similar to previous ones of Griffiths et al. (2000), who observed that community respiration was inversely correlated with biodiversity, while the relationship was positive for specific functions such as nitrification in soil microcosms with reduced diversity. A similar study using a single trait-centered approach (Philippot et al., 2013), found that a removal of 75% of the denitrifiers OTUs produced a decrease in potential denitrification activity of about 48 to 88%. This suggests that more specific functions have even a lower degree of functional redundancy than those tested in this experiment, and therefore are more sensitive to a loss of biodiversity.

The relationship between diversity and richness and utilisation of glucose seems not to be following any clear trend. The ability of utilising glucose is a universal function that the majority of microbes is able to carry out. In this experiment, the loss of diversity did not impair the ability of the remaining species to degrade this compound, possibly due to the redundancy associated with this function (i.e. several species able to use glucose as substrate are present in the community).
According to the insurance hypothesis (Yachi and Loreau, 1999) a high biodiversity would confer an ‘insurance’ against ecosystem malfunctioning under stress or disturbance. Our findings do not comply with the insurance hypothesis, but rather we have shown that a reduction in diversity has the ability to reduce both an aggregate and a specific ecosystem function. Although it is correct that an aggregate function is carried out by many different species, it is also valid that different species will perform better due to niche partitioning (Cardinale et al., 2011), selection and facilitation effects (Loreau and Hector, 2001). The selection effect might have also played a role, as removal of key species might have occurred when diluting the cell suspensions. But for a broad function the selection effect is probably not the central factor in determining the gradient of function observed. We suggest that for the system and design tested in this work the complementary effect played the most important role: a high number of species resulted in a higher number of genes, enzymes, pathways and resources that facilitated the usage of a natural pool of carbon sources and hence an augmented respiration rate (Bell et al., 2005). With the addition of glucose to the media, the microbial communities might have addressed their metabolic efforts to this more easily available compound, resulting in a change in the BEF relationship.

Overall, these results, together with the literature reviewed above, suggest that specific functional parameters may represent a more sensitive indicator of environmental change than general parameters.

An increase in respiration rates between the undiluted treatment and the $10^{-2}$ dilution was observed in the control and with addition of carbon substrates (Figure 2.4). This might have occurred as a consequence of decreased competition due to the dilution...
of the original community, which would have lead to increased accessibility to the resources, resulting in a higher level of respiration, that was observed also for the specific function.

2.4.3. Microbial diversity and functional stability

The relationship between microbial diversity and functionality in the context of perturbation has been addressed in a few studies that used serial dilutions of soil microbial communities as models (Griffiths et al., 2000, 2004, Wertz et al., 2007). These studies found no consistent connection between biodiversity and the stability (resistance and resilience) of both broad and specialised functions, attributing that to the high functional redundancy of the microbial communities.

In the study reported here, the disturbance applied had different effects on the resistance of the community depending on the function considered. The resistance index calculated on community respiration had values significantly lower ($P < 0.05$) in more diluted communities ($10^{-6}$ dilution) than in less diluted ones ($10^{-2}$ dilution). This indicates that the decrease in diversity affected the ability of the microbial communities to resist a transient disturbance. On the other hand, we did not observe any significant difference in the resistance index across dilutions for the utilisation of both glucose and lignin. Because utilisation of glucose is a universal function among bacterial groups, the loss of abundance or changes in the community composition that the disturbance might have caused did not impair the ability of the species that survived the disturbance to utilise this labile compound. This shows, once again, the potential functional redundancy of the community for glucose utilisation. In the case of lignin, the high values of the resistance index in the high dilutions might be
explained by the fact that lignin is mainly degraded by actinomycetes (Zimmermann, 1990, Bugg et al., 2010). Since members of this group are heat tolerant (Jensen et al., 1991, Takizawa et al., 1993), they might have survived the disturbance occupying the niche left free by the non-tolerant groups, without changing the overall respiration rate and, therefore, resulting in high values of the resistance index. This is consistent with Goldfarb et al. (2011), who found that only six taxa of soil bacteria were stimulated by the addition of lignin but not by other substrates. It is therefore possible that these groups did not contribute to the basal respiration rates, but they positively reacted to lignin amendment. Therefore, in this case, species composition could explain better then diversity or richness the changes in resistance across dilution treatments.

Some studies (e.g. Müller et al., 2002) showed that functional performance of soil microbial communities was unaltered by a transient disturbance, whereas it was more sensitive to an additional disturbance. In our study, the microcosms were subjected to a single perturbation. The resistance index showed quite high values (> 0) in all the dilutions for the functions tested, thus indicating that the disturbance did not change greatly the functioning of the communities. Despite this, none of the communities fully recovered their functionality to the levels of the control, as the values of the resilience index were close to zero or lower in all the dilutions and all functions. The resilience indices for each function appeared lower in the dilution treatments than in the undiluted community, which could indicate that the reduction of diversity impaired the ability of the communities to recover their functioning after disturbance, although these differences were not statistically significant. These results demonstrate that the reduction of microbial communities’ diversity and
richness via dilution-to-extinction approach led to lower functional resistance and resilience.

2.5. Conclusions and future directions

For microbial communities, previous biodiversity-ecosystem functioning experiments have shown contrasting results, and it is commonly thought that microbial communities are highly redundant (Wertz et al., 2006). In this work, a significant reduction in bacterial richness achieved by a dilution-to-extinction approach created a significant reduction in carbon mineralisation. OTU richness was correlated to the functions investigated in this study by linear relationships. The slope of the BEF relationship depended on the function considered. In fact, I observed the strongest relationship for the specialised function (i.e. lignin utilisation) followed by basal respiration, while glucose utilisation was characterised by functional redundancy. This highlights the importance of considering multiple functions in BEF studies: considering only one function, in fact, might over- or underestimate the functional redundancy of the system. The use of a wider range of carbon substrates in this experiment could give deeper insights into the extension of redundancy under a multifunctional perspective.

I also observed effects of the dilution approach on resistance and resilience of the system, but it was not possible to find any linear relationship between stability and microbial diversity in this work. Nonetheless, this study suggests that the loss of diversity in soil systems has the potential to affect their functional stability to perturbations. However, the boundaries of sustainable losses of microbial diversity still need to be assessed, particularly including the study of the role of fungal
diversity in soil functioning. The use of more sensitive measures of microbial diversity, such as high-throughput sequencing, has the potential to detect stronger relationships with functioning, and would also allow investigating the effects of dilution on community composition and the link with processes.

For conservation decisions, these results emphasise that the loss of microbial diversity may have stronger effects on the performance of specialised functions, while it may not affect significantly general functions, due to the redundancy of microbial communities for these functions. Whether the results of this laboratory study will match the dynamics of “real-world ecosystems” needs to be verified by means of field experiments, taking into account the role played by soil structure and spatial distribution of microorganisms at a micro scale.
CHAPTER 3

Effects of intensive management on the link between soil microbial communities and ecosystem functioning in a dryland farming system
3.1. Introduction

Soil microorganisms facilitate numerous essential soil processes, including regulation of biogeochemical cycling of nutrients and soil aggregate stability, organic matter formation and decomposition, and water regulation. All of these processes are of fundamental importance for agroecosystem functioning and plant productivity (Köhl et al., 2014). It is known that more biodiverse ecosystems show higher nutrient use efficiency and disease suppressiveness (Bardgett and McAlister, 1999, van Elsas et al., 2002) and better overall soil performance (Eisenhauer et al., 2010, Wagg et al., 2011). Consequently, the conservation of soil biological diversity is necessary to ensure sustainable land use and the provision of ecosystems services and functions.

Agricultural practices such as tillage, fertilisation, and crop rotation, have significant impacts on soils and soil biota (Bünemann et al., 2006, Postma-Blaauw et al., 2010). In particular, management intensity and fertilisation are known to significantly alter bacterial diversity and community composition (Jangid et al., 2008, Culman et al., 2010). In a study comparing different agricultural management systems, higher bacterial richness, evenness, and biomass were observed in pastures than in croplands (Jangid et al., 2008). Also the community composition was observed to differ according to management intensity (Jangid et al., 2008).

Nitrogen inputs used in agriculture to improve pasture and crop productivity can lead to soil acidification and consequently reduce the buffering capacity and lead to accumulation of toxins, which negatively impact soil microbial diversity and activity (Lauber et al., 2009, Liu et al., 2014). Changes in soil bacterial communities due to altered resource availability caused by fertilisation may influence ecosystem
processes by altering bacterial functions. Several studies found long-term N fertilisation effects on bacterial community compositional shifts and subsequent alteration of ecosystem functions, such as decomposition, potential ammonia oxidation and denitrification (Hallin et al., 2009, Fierer et al., 2011, Ramirez et al., 2012). Since increasing N inputs increase plant biomass and dissolved organic carbon, microbial activities are expected to increase with the increase in carbon supplied by plants (Liu et al., 2014). However, many studies demonstrated reductions in soil microbial biomass and respiration under N enrichment (Treseder, 2008, Liu and Greaver, 2010, Ramirez et al., 2012, Liu et al., 2014). Agricultural management may impact soil biota also through the manipulation of aboveground diversity. In fact, the richness and structural community composition of soil microorganisms depend on plant species and plant functional groups (König et al., 2010, Millard and Singh, 2010), and have been shown to differ among the rhizospheres of different crop species (Ibekwe and Kennedy, 1998, Wieland et al., 2001) and pasture type (Wakelin et al., 2009). Because plant species differ in the quantity and quality of resources that they transfer to soil, individual plant species can influence soil microbial communities and the processes they regulate (Wardle et al., 2004).

Since agricultural systems are subject to a range of practices that disrupt soil biogeochemical cycles and structure, they provide an excellent opportunity to investigate the roles of land management in shaping microbial communities and ecosystem functioning. In this study, the microbial communities and soil functions involved in decomposition were investigated in an experimental pasture-cropping system. Pasture cropping is a term applied to a farming system in which annual crops
and summer-active pastures are grown together. This system is becoming more common in the Northern Agricultural Region (NAR) of Western Australia (WA). This region is, in fact, dominated by sandy soils of low fertility that usually support sparse annual pasture and are only occasionally cropped. Because of their low water holding capacity, productivity and ground cover, these soils are subject to wind erosion, groundwater recharge and associated dryland salinity (Ward et al., 2012). Therefore, farmers in the region are establishing sub-tropical perennial grasses on these marginal sandy soils, in an effort to stabilise the soils and produce forage (Lawes and Robertson, 2008). During winter, when the sub-tropical perennials become dormant, a crop is grown with the benefits of maintaining ground cover, reducing dryland salinity and soil erosion, and producing a crop from marginal soils. In 2008, an agricultural experiment was established 20 km south-west of Moora (WA) to test different sub-tropical species (Gatton panic, a tufted grass; Rhodes grass, a creeping grass; and Siratro, a perennial legume) for their suitability for pasture cropping on the marginal sandy soils in the NAR. Soils were sampled within this experimental site to explore the effects of the pasture-cropping practice on the microbial communities and their relationship with soil functioning. It is hypothesised that different plant assemblages (crop monoculture, pasture, pasture-crop) subjected to different land management (nitrogen amendments), would harbour soil microbial communities with different diversity levels and composition. The aim of this study was to investigate the relationship between microbial diversity and soil ecosystem functioning in an arid agricultural system subjected to pasture-cropping management practices. Bacterial diversity and soil processes were expected to be enhanced under pasture-cropping treatments because of the positive effects due to the presence of
two different plant species, and affected by nitrogen treatment because of its negative effects on soil microbial diversity and activity.

3.2. Materials and Methods

3.2.1. Site description and soil sampling

The selected site is located 20 km south west of Moora (WA, 31° S, 116° E) on a yellow deep sandy moderately acidic soil (Ward et al., 2012). Perennial pastures Gatton panic (*Megathyrsus maximus*), Katambora Rhodes grass (*Chloris gayana*) and Aztec Siratro (*Macroptilium atropurpureum*) were sown in September 2008. Row spacing was 36 cm for Siratro and Rhodes grass, and 36 or 72 cm for Gatton panic. Crops of Buloke barley were sown in 2009 and 2011 in the ‘crop only’ and ‘pasture cropped’ plots. A ‘pasture only’ control was included. Pasture crop and crop only were subjected to two nitrogen treatments: 50 kg N/ha (low) and 80 kg N/ha (optimal) for a total of 14 different treatments with three replicates (Ward et al 2012). All the crops and treatments were maintained through to April 2014. For the present study only the plots with 36 cm row spacing were sampled, for a total of 11 treatments in 3 replicated plots (Table 3.1). Each plot measured 6 x 30 metres. Five soil cores (2 cm in diameter and 10 cm deep) were collected between rows within the central area of each plot, after the crops had been harvested in April 2014. The subsamples were pooled and homogenized into a composite sample for each plot, sieved through a 2 mm mesh, and stored in plastic bags at 4 °C in the dark until analyses.
Table 3.1 List of treatments at the Moora pasture cropping site.

<table>
<thead>
<tr>
<th>Treatment ID</th>
<th>Treatment description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNH, CNL</td>
<td>Crop only, with optimum (CNH) or low N (CNL)</td>
</tr>
<tr>
<td>G</td>
<td>Gatton panic only</td>
</tr>
<tr>
<td>GCNH, GCNL</td>
<td>Gatton panic, pasture cropped with optimum (GCNH) or low N (GCNL)</td>
</tr>
<tr>
<td>R</td>
<td>Rhodes grass only</td>
</tr>
<tr>
<td>RCNH, RCNL</td>
<td>Rhodes grass, pasture cropped with optimum (RCNH) or low N (RCNL)</td>
</tr>
<tr>
<td>S</td>
<td>Siratro only</td>
</tr>
<tr>
<td>SCNH, SCNL</td>
<td>Siratro, pasture cropped with optimum (SCNH) or low N (SCNL)</td>
</tr>
</tbody>
</table>

3.2.2. Determination of relative bacterial diversity and richness

Total genomic DNA was isolated from 0.25 g of soil using PowerSoil® DNA Isolation kit (MoBio Laboratories, CA, USA) according to manufacturer’s instructions, with modifications that a FastPrep bead beating system (Bio-101, Vista, CA, USA) at a speed of 5.5 m s⁻¹ for 30 seconds was used.

The bacterial community was explored using 16S amplicon sequencing. The 16S rRNA gene region V3-V4 was sequenced on an Illumina MiSeq sequencer (Illumina Inc. San Diego, CA, USA) by the Next Generation Sequencing Service at University of Western Sydney (Richmond, NSW, Australia). Paired-ends reads of 312 bp were obtained using the primers 341F (5’-CCTACGGGNGGCWGCAG-3’) and 805R (5’-GACTACHVGGGTATCTAATCC-3’) (Herlemann et al., 2011). Data analysis was performed using the ‘Quantitative Insights Into Microbial Ecology’ (QIIME v
1.8.0) software package (Caporaso et al., 2010). Paired ends were joined using the ‘SeqPrep’ method. Barcode, linker primer and reverse primer sequences were removed from the raw sequence reads using the ‘split_libraries.py’ script while setting minimum quality score of 20. Sequences were then screened for the presence of chimeric sequences using USEARCH (Edgar, 2010, Edgar et al., 2011). Similar sequences were binned into operational taxonomic units (OTUs) using ‘UCLUST’ method (minimum pairwise identity of 97%, Edgar, 2010). Taxonomic designations of OTUs were made using Greengenes, as described by McDonald et al (2011) and Werner et al. (2011). The number of sequences per sample was rarefied to the lowest number of sequences (i.e. 16 876) to ensure even sampling depth across all samples. OTU richness and diversity indices were then calculated on relative abundance OTU tables with the equations given in section 2.2.5.3. The relative abundance OTU table was also used for multivariate analysis of community composition.

3.2.3. Quantification of bacterial and fungal abundances

The relative abundances of total bacteria and fungi were assessed by means of quantitative PCR (qPCR) of 16S rRNA gene (bacteria) and 18S rRNA gene (fungi). qPCR assays were conducted in 96-well plates on a CFX96 Touch™ Real-Time PCR Detection System (BioRad, Australia). Each 10µl-reaction contained 5 µl of SensiFAST™ SYBR no-ROX Master mix (Bioline, Australia), 0.1 µl bovine serum albumin (BSA, 20 mg/ml, New England Biolabs, USA), 0.8 µl nuclease-free water (Ambion, Life Technologies, Australia), bacterial primers 338F and 518R or fungal primers Fung5F and FF390R (SigmaAldrich, Australia, primer sequences listed in Table 3.2) at a final concentration of 250 nM, and 4 µl of DNA. The PCR conditions were 95 °C for 3 minutes followed by 40 cycles of 95 °C for 15 seconds, 53 °C
(bacteria) or 57 °C (fungi) for 15 seconds, and 72 °C for 30 seconds. Fluorescent signals were measured after this last step at each cycle. Each plate included duplicate reactions per DNA sample and per the appropriate set of standards and negative control samples (without DNA).

**Table 3.2** Oligonucleotides used for the amplification of bacterial and fungal ribosomal DNA in qPCR reactions.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Target region and specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eub338F</strong></td>
<td>ACTCCTACGGGAGGCAGCAG</td>
<td>16S rRNA gene Eubacteria</td>
<td>(Amann et al., 1990)</td>
</tr>
<tr>
<td><strong>Eub518R</strong></td>
<td>ATTACCGCGGTGCTGG</td>
<td>16S rRNA gene Eubacteria</td>
<td>(Muyzer et al., 1993)</td>
</tr>
<tr>
<td><strong>Fung5F</strong></td>
<td>GTAAAAGTCCTGGTCCCCC</td>
<td>18S rRNA gene Fungi</td>
<td>(Smit et al., 1999)</td>
</tr>
<tr>
<td><strong>FF390R</strong></td>
<td>AGGTCTCGTTCGTTATCG</td>
<td>18S rRNA gene Fungi</td>
<td>(Vainio and Hantula, 2000)</td>
</tr>
</tbody>
</table>

Melt curve analysis of the PCR products was conducted after each assay (ramp from 65 to 95 °C, rising by 0.5 °C each step) to confirm that the fluorescence signal was originated from specific PCR products and not from primer dimers or other artefacts (BioRad CFX Manager 3.0 Software, Australia).

A plasmid standard containing the target region was generated for each primer set using DNA extracted from the appropriate positive control strain, following the procedure reported in section 2.2.4. Seven standard concentrations per assay were included, with standard concentration ranging from $10^9$ to $10^3$ copies μl$^{-1}$. Target copy numbers for each reaction were calculated from the standard curves by means of the CFX Manager 3.0 Software (BioRad, Australia) and expressed as copies per gram of soil.
3.2.4. Functional measurements

3.2.4.1. Potential enzyme activity

The potential activities of extracellular enzymes involved in carbon and nutrient cycling were measured using fluorimetric assays. Three enzymes were assayed: β-glucosidase (BG), which hydrolyzes glucose monomers from cellulose, N-acetyl-glucosaminidase (NAG), which releases amino sugars from chitin, and acid phosphatase (AP), which cleaves phosphate groups from organic phosphorous. These three enzymes were chosen because they are involved in C, N and P cycling, respectively. Synthetic substrates linked to a fluorescent dye, 4-methylumbelliferyl, were used. The enzyme-catalyzed substrate degradation releases the fluorescent dye producing fluorescence, which is used as an indirect assessment of enzyme activity. All substrate analogues were obtained from Sigma Aldrich (Australia) in a crystalline form. A list of the substrates that allow the targeting of the abovementioned enzymes is given in Table 3.3.

Table 3.3 Substrates used in extracellular enzyme assays

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>MUB-linked substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-glucosidase (BG)</td>
<td>Cellulose</td>
<td>4-methylumbelliferyl β-D-glucopyranoside</td>
</tr>
<tr>
<td>N-acetyl-glucosaminidase (NAG)</td>
<td>Chitin</td>
<td>4-methylumbelliferyl N-acetyl-β-D-glucosaminide</td>
</tr>
<tr>
<td>Acid phosphatase (AP)</td>
<td>Organic phosphorous</td>
<td>4-methylumbelliferyl phosphate</td>
</tr>
</tbody>
</table>

The substrates were pre-dissolved in 1 ml of methoxyethanol (Sigma Aldrich, Australia) and then made up to stock solutions of 10 mM with sterile deionised
water. New working solutions of the enzyme substrates were prepared for each assay by diluting the stock solutions to 1 mM in sterile 15 ml centrifuge tubes. All dilutions were made in autoclaved deionised water (Marx et al., 2001). A standard curve of fluorescence intensities was made in order to calculate the enzyme activity, taking into account fluorescence quenching by soil particles. A 4-Methylumbelliferone (MUB, Sigma Aldrich, Australia) solution was used as the fluorescence standard and made by pre-dissolving the powder in 1 ml acetone and then diluting it in sterile deionised water to make up the appropriate concentrations (see below).

One gram of each fresh soil sample (2 mm sieved) was homogenised in 125 ml 50 mM sodium acetate buffer in a sonicating bath (Soniclean™, Australia) at maximum power for 2 minutes. Buffer pH was adjusted with the addition of acetic acid to the mean pH value of study soils (7.02 ± 0.26). The resulting slurry was loaded in a Nunc™ 96-well black microplate (Thermo Fisher, Australia), in the volume of 200 µl per well. Each substrate was added in 50 µl aliquots to eight separate replicated wells, each with the final concentration of 200 µM. This non-limiting substrate concentration was chosen after an optimisation trial (see Appendix C). Microplates were incubated in the dark at 25 °C for one hour. A standard curve was prepared for each sample by adding standard to the soil slurry to give final amounts of 0, 10, 20, 30, 40, 50, 60, 70 pmol MUB well⁻¹.

Fluorescence was measured at 365 nm excitation and 450 nm emission on a spectrophotometer microplate reader (EnSpire® 2300 Multilabel Reader, Perkin Elmer, USA) immediately after adding 10 µl 1 M NaOH to inhibit the reaction and maximise the fluorescence intensity (Hoppe, 1983, Freeman et al., 1995).
Fluorescence was converted to potential enzyme activity in nmol g\(^{-1}\) soil dry weight h\(^{-1}\) based on the MUB standard curve as reported by Bell et al. (2013).

3.2.4.2. Community respiration and substrate-induced respiration

MicroResp\(^{\text{TM}}\), as described by Campbell et al. (2003), was used to measure community respiration and community level physiological profiles (CLPP). Soil samples were placed in the 96- deep well plates, delivering approximately 0.4 g of soil to each well. The microplates were incubated for two days at field moisture and at room temperature in the dark before the assay was carried out. A total of 11 different carbon substrates, consisting of four amino acids, two carbohydrates, three carboxylic acids, and two complex compounds (polymers and containing benzene rings) were selected (Table 3.4). Substrates were chosen based on their complexity (i.e. length of the C chain). This approach is based on the assumption that differences in the respiration responses to supplied C substrates between soils represent a difference in C mineralization capacity. All the C sources were obtained from Sigma Aldrich (Australia). Substrates were supplied at 30, 15 or 1 mg ml\(^{-1}\) of water contained by the soils, depending on the solubility of the compound in water (Table 3.4), to four replicated wells. Substrates were dissolved in deionised water and filter sterilised.

Sterile deionised water was delivered to four replicate wells to measure basal respiration. Immediately prior to addition of C sources, CO\(_2\) detection plates were read (T0) and then the deep-well plates were sealed with the pre-read CO\(_2\) detection plates and incubated at 25 °C for 6 hours in the dark, as recommended by the manufacturer (Macaulay Scientific Consulting, UK).
Table 3.4 The 11 C substrates used in the MicroResp™ assay, their classification and number of carbon (C) and nitrogen (N) atoms in their molecule.

<table>
<thead>
<tr>
<th>Carbon substrates</th>
<th>Classification</th>
<th>Supply concentration (mg ml⁻¹ soil water)</th>
<th>C</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Amino acid</td>
<td>15</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>L-cysteine hydrochloride</td>
<td>Amino acid</td>
<td>15</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Gamma aminobutyric acid</td>
<td>Amino acid</td>
<td>15</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Lysine</td>
<td>Amino acid</td>
<td>15</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Glucose</td>
<td>Carbohydrate</td>
<td>30</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>N-acetyl glucosamine</td>
<td>Carbohydrate</td>
<td>15</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Oxalic Acid</td>
<td>Carboxilic acid</td>
<td>30</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Malic Acid</td>
<td>Carboxilic acid</td>
<td>30</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>α-ketoglutaric acid</td>
<td>Carboxilic acid</td>
<td>30</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Lignin</td>
<td>Aromatic alcohol</td>
<td>1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>Dihydroxybenzoic acid</td>
<td>1</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

The change in optical density was then measured on a spectrophotometer microplate reader (EnSpire® 2300 Multilabel Reader, Perkin Elmer, USA) at a wavelength of 570 nm. The rate of CO₂ respiration expressed per gram of soil per well was calculated using the formula provided in the MicroResp™ manual (Macaulay Scientific Consulting, UK), which accounts for differences in field moisture across soil samples:

$$\text{CO}_2 \text{ g}^{-1} \text{dry soil} = \frac{\left(\frac{\% \text{CO}_2}{100}\right) \times \text{headspace volume} \times \left(\frac{44}{22.4}\right) \times \left(\frac{12}{44}\right) \times \left(\frac{273}{273 + 25^\circ \text{C}}\right)}{\text{soil fresh weight} \times (\text{soil \%} \ \text{dry weight}) \times \text{incubation time}}$$
The total substrate respiration responses were calculated by subtracting the water response from each individual substrate response and then summing the single responses for each sample. The subtraction of the water response from individual substrate response was meant to consider the initial flush of CO₂ evolution produced when a dry soil is moistened, and to separate the effects of water addition from the those of substrate addition.

3.2.5. Soil physicochemical analyses

3.2.5.1. Exchangeable nitrate and ammonium

The extraction of nitrate (NO₃⁻) and ammonium (NH₄⁺) was performed by mixing 5 ± 0.01 g of fresh soil sieved through a 2-mm mesh in 50 ml 2 M KCl solution (Keeney and Nelson, 1982). This was mixed on a shaker for 60 minutes at 150 r.p.m. and allowed to settle for 20 minutes after shaking. The solution was then filtered through Whatman No. 42 filters and analysed with a SEAL AQ2 Analyzer (SEAL Analytical, Maquon, WI, USA).

3.2.5.2. Extractable phosphate

The extractable phosphate (PO₄³⁻) was extracted from sieved air-dried soil (40 °C for 48 hours) following the Bray method (Bray and Kurtz, 1945). 4.28 ± 0.01 g of soil was mixed with Bray’s reagent (0.03 M NH₄F and 0.025 M HCl). The samples were then shaken for 1 minute at 170 r.p.m. on a horizontal shaker and filtered through Whatman No. 42 filters. The concentration of extractable PO₄³⁻ was then analysed with a SEAL AQ2 Analyzer (SEAL Analytical, Maquon, WI, USA).
3.2.5.3. Total carbon and nitrogen

Total carbon (C) and nitrogen (N) in the soils were obtained following the Dumas combustion method. Five grams of each soil sample were oven dried at 40 °C for approximately 48 hours. The soils were then milled to a fine powder (< 0.5 mm) by using a Mixer Mill MM400 grinder (Retsch, Germany). Two hundred µg of each sample were analysed on a LECO macro-CN analyzer (LECO, St Joseph, MI, USA) using a synthetic standard (%C: 2.35 ± 0.07; %N: 0.183 ± 0.010).

3.2.5.4. Soil pH

To measure soil pH, 2 ± 0.01 g of each fresh sample were mixed to 10 ml of deionised water. The mix was then vortexed for 5 seconds and allowed to settle for 30 minutes. This step was repeated twice and the pH was then read with a Delta pH-meter (Mettler-Toledo Instruments, Columbus, OH, USA).

3.2.5.4. Soil moisture content

Soil moisture content was determined by measuring the difference of weight of soil samples before and after oven drying at 105 °C for 24 hours, and expressed as percentage of soil weight.

3.2.6. Statistical methods

All the statistical analyses were carried out using the software R 3.1.1 (R Core Development Team 2014). Bacterial community composition (relative OTU abundance data) was visualised using principal coordinates analysis (PCoA, Gower, 1966) based on Manhattan dissimilarity index. The significance of treatment effect was assessed by using a two-way Permutational Multivariate Analysis of Variance.
(PerMANOVA, Anderson, 2001), with vegetation type (i.e. crop only, pasture only, and pasture crop), nitrogen treatment and their interaction as factors. Bacterial community data were also explored using Manhattan distance-based redundancy analysis (dbRDA, Legendre and Anderson, 1999) to reveal relationships between bacterial communities and the soil physico-chemical properties measured. The edaphic variables that significantly influenced the bacterial communities were identified by a backward stepwise selection method using permutation tests (Blanchet et al., 2008). All the bacterial community data analyses were performed using the R package ‘vegan’ (Oksanen et al., 2014).

The data of diversity indices (i.e. OTU richness, Simpson’s diversity index, Pielou’s index of evenness), bacterial abundances, potential enzyme activity and respiration were analysed using a two-way ANOVA, with vegetation type, nitrogen treatment and their interaction as explanatory variables. The dependent variables were tested for normality and homoscedasticity using the Shapiro-Wilk’s test and the Bartlett’s test, respectively. Bacterial 16S rRNA and fungal 18S rRNA gene copies were log- and rank-transformed respectively to meet the homoscedasticity and normality assumptions of the ANOVA. Where significant differences for the main effect were observed ($P < 0.05$), a Tukey’s pairwise comparison test was also performed.

Because pasture-only treatments were not subjected to nitrogen amendment, they were considered only as vegetation type by the two-way analyses (i.e. PerMANOVA and ANOVA).

Soil functioning was analysed by means of stepwise multiple regressions examining the explanatory power of microbial community variables and edaphic factors, following a method developed by Diaz et al. (2007) for plant communities and
modified by Powell et al. (2015) for microbial communities. The predictor variables were classified into four groups: 1) diversity components (i.e. richness, diversity and evenness), 2) microbial abundances (i.e. bacterial and fungal abundances), 3) bacterial community composition, and 4) abiotic environmental variables. Fungal:bacterial ratio was initially selected as a component of the second class, but, because it did not result in a significant predictor of any of the function, and models had a better fit when it was not included, the ratio was excluded from the analysis.

For the third class, pairwise correlations were performed between data of each function and the relative abundance or presence/absence of specific OTUs, employing the Šidák correction for multiple comparisons (Šidák, 1967). When the response variables were not correlated to any OTUs, the PCoA scores of the first two axes were used as predictors in this class. For each response variable, a linear model was fit separately with all the predictor variables of each class. The variables that were statistically significant predictors of the response variable in this first step were combined into a single linear model that explained variation in the response variable. The significance of each predictor was assessed based on the contribution to the model fit. The model fit was assessed using the Akaike Information Criterion (AIC), corrected for small sample size (AICc; Hurvich and Tsai, 1989), with lower values of AICc indicating better model fits. Akaike weights (Burnham and Anderson, 2002) were calculated for the final model including all the significant variables, and then for models in which the variables were excluded one by one, and for intercept-only models. Data were inspected for non-linear relationships between the response and the predictor variables.
3.3. Results

3.3.1. Soil properties

The physico-chemical properties of soils at Moora experimental site are reported in Table 3.5. The treatments significantly influenced soil pH ($F_{10,22} = 3.1, P = 0.012$), which was 6% lower in CNL and RCNH plots compared to G plots. No effect of treatment was observed on the rest of the plots, which showed similar pH values (Table 3.5). Ammonium ($NH_4^+$) was found to have the highest concentrations in GCNL soils and the lowest in R soils (76% lower), which were significantly different ($F_{10,22} = 2.339, P = 0.046$). The other soil properties analysed were not observed to be influenced by pasture cropping treatments. Soil moisture was very low in all the soils, with values ranging from 0.38 to 1.80% and no significant difference across treatments ($F_{10,22} = 0.782, P = 0.644$). Total carbon and total nitrogen ranged from 8.18 to 18.74 g/kg and from 0.54 and 1.45 g/kg of soil respectively and were not significantly different across treatments ($F_{10,22} = 1.035, P = 0.448$; $F_{10,22} = 1.007, P = 0.467$ respectively). No effect of treatment was observed on the concentration of phosphate ($F_{10,22} = 1.326, P = 0.277$), which ranged from 2.21 to 19.42 mg/kg of soil, and nitrate ($F_{10,22} = 0.910, P = 0.540$), which ranged from 0.47 to 4.77 mg/kg of soil.
Table 3.5 Mean values of soil pH, soil moisture content, total carbon, total nitrogen, phosphate concentrations (PO$_4^{3-}$), ammonium concentrations (NH$_4^+$), nitrate concentrations (NO$_3^-$) across different treatments. CNH = crop only with optimum N; CNL = crop only with low N; G = Gatton panic only; GCNH = Gatton panic and crop with optimum N; GCLH = Gatton panic and crop with low N; R = Rhodes grass only; RCNH = Rhodes grass and crop with optimum N; RCNL = Rhodes grass and crop with low N; S = Siratro only; SCNH = Siratro and crop with optimum N; SCNL = Siratro and crop with low N. Different letters indicate values that are significantly different ($P < 0.05$). Values in parenthesis represent ± one standard error (n = 3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH</th>
<th>Moisture</th>
<th>Total C (g kg$^{-1}$)</th>
<th>Total N (g kg$^{-1}$)</th>
<th>PO$_4^{3-}$ (mg kg$^{-1}$)</th>
<th>NH$_4^+$ (mg kg$^{-1}$)</th>
<th>NO$_3^-$ (mg kg$^{-1}$)</th>
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</thead>
<tbody>
<tr>
<td>CNH</td>
<td>6.99$^b$</td>
<td>0.91 (0.42)</td>
<td>14.02 (2.22)</td>
<td>0.95 (0.15)</td>
<td>5.18 (0.45)</td>
<td>4.73$^b$ (1.46)</td>
<td>2.58 (0.65)</td>
</tr>
<tr>
<td>CNL</td>
<td>6.86$^b$</td>
<td>0.99 (0.41)</td>
<td>9.28 (0.59)</td>
<td>0.66 (0.03)</td>
<td>8.27 (1.64)</td>
<td>6.89$^b$ (3.85)</td>
<td>2.69 (1.00)</td>
</tr>
<tr>
<td>G</td>
<td>7.28$^a$</td>
<td>0.85 (0.13)</td>
<td>12.82 (0.96)</td>
<td>0.89 (0.05)</td>
<td>12.98 (1.25)</td>
<td>4.38$^b$ (1.70)</td>
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<tr>
<td>GCNH</td>
<td>7.10$^b$</td>
<td>0.38 (0.29)</td>
<td>12.52 (2.36)</td>
<td>0.87 (0.17)</td>
<td>11.18 (2.75)</td>
<td>10.45$^b$ (3.23)</td>
<td>3.19 (1.02)</td>
</tr>
<tr>
<td>GCNL</td>
<td>7.00$^b$</td>
<td>0.58 (0.002)</td>
<td>12.98 (1.52)</td>
<td>0.93 (0.11)</td>
<td>10.16 (3.09)</td>
<td>12.45$^a$ (0.33)</td>
<td>2.24 (0.37)</td>
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<tr>
<td>R</td>
<td>7.08$^a$</td>
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<td>12.00 (0.96)</td>
<td>0.85 (0.01)</td>
<td>11.42 (1.25)</td>
<td>2.95$^b$ (0.40)</td>
<td>2.05 (0.40)</td>
</tr>
<tr>
<td>RCNH</td>
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<td>0.72 (0.06)</td>
<td>13.04 (0.68)</td>
<td>0.88 (0.04)</td>
<td>8.62 (3.15)</td>
<td>3.68$^b$ (0.04)</td>
<td>1.73 (0.73)</td>
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<td>RCNL</td>
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<td>0.66 (0.26)</td>
<td>13.46 (1.74)</td>
<td>0.93 (0.11)</td>
<td>14.69 (2.64)</td>
<td>4.58$^b$ (0.95)</td>
<td>1.97 (0.58)</td>
</tr>
<tr>
<td>S</td>
<td>7.06$^b$</td>
<td>0.92 (0.29)</td>
<td>12.76 (0.44)</td>
<td>0.91 (0.05)</td>
<td>10.83 (1.75)</td>
<td>6.64$^b$ (2.50)</td>
<td>2.06 (0.23)</td>
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<tr>
<td>SCNH</td>
<td>6.95$^b$</td>
<td>1.10 (0.33)</td>
<td>10.60 (0.97)</td>
<td>0.71 (0.05)</td>
<td>10.58 (3.17)</td>
<td>5.69$^b$ (1.93)</td>
<td>2.92 (0.68)</td>
</tr>
<tr>
<td>SCNL</td>
<td>6.97$^b$</td>
<td>0.71 (0.12)</td>
<td>12.55 (0.58)</td>
<td>0.91 (0.02)</td>
<td>6.80 (3.27)</td>
<td>3.80$^b$ (0.32)</td>
<td>2.81 (0.69)</td>
</tr>
</tbody>
</table>
3.3.2. Community composition and bacterial diversity

Between 16,879 and 45,904 unique sequences were obtained after quality filtering, with an average of 33,292 sequences per sample. These were subsequently clustered into 42,172 OTUs.

The treatments applied at Moora experimental site influenced the bacterial community composition (Figure 3.1), with a significant effect of the nitrogen treatments (i.e. low, optimal and control in the pasture-only plots, $P = 0.001$), that explained about 15% of the variance, and a marginally non-significant effect of the interaction between vegetation and nitrogen treatments ($P = 0.052$), explaining 11% of variance. The vegetation treatment alone (i.e. pasture only, pasture-crop, and crop only) did not have significant effects ($P = 0.370$).
Figure 3.1 Ordination plot of the first two axes (explaining 42.56 and 20.46% of the variance, respectively) produced by principal coordinates analyses of Manhattan distances of bacterial community composition, based on amplicon 16S rRNA gene data. In the legend ‘N’ refers to nitrogen treatment: C = control (no N amendment); H = optimum N; L = low N. ‘Veg’ refers to vegetation treatment: C = crop only; G = Gatton panic only; GC = Gatton panic and crop; R = Rhodes grass only; RC = Rhodes grass and crop; S = Siratro only; SC = Siratro and crop.

Soil pH was the main environmental factor influencing the bacterial community, as identified by the dbRDA (Figure 3.2), since it was found to be significantly associated with bacterial community composition ($P = 0.005$). The total amount of variance explained by the edaphic properties measured was 29.17%.
Figure 3.2 Ordination plot of the first two axes produced by redundancy analysis based on Manhattan distances. Effects of edaphic variables on bacterial community composition, based on amplicon 16S rRNA gene data. In the legend ‘N’ refers to nitrogen treatment: C = control (no N amendment); H = optimum N; L = low N. ‘Veg’ refers to vegetation treatment: C = crop only; G = Gatton panic only; GC = Gatton panic and crop; R = Rhodes grass only; RC = Rhodes grass and crop; S = Siratro only; SC = Siratro and crop.

A two-factor analysis of variance showed significant effects of vegetation on OTU richness ($F_{6,22} = 4.018, P = 0.007$). Gatton panic-only plots (G) had higher OTU richness than crop only (CNL, CNH), Rhodes grass and crop (RCNH, RCNL) and Siratro grass and crop (SCNH, SCNL) plots. A significant effect of nitrogen treatment was observed ($F_{1,22} = 14.227, P = 0.001$), with soils treated with nitrogen at an optimal concentration showing lower OTU richness than soils treated with nitrogen at a low concentration. The interaction between vegetation and nitrogen treatments was also significant ($F_{3,22} = 4.390, P = 0.015$, Figure 3.3A). Gatton panic-
only plot (G) had the highest OTU richness (409 ± 5.17 OTUs), which was significantly higher than CNL, RCNH, and SCNH treatments. A significant difference was observed also between SCNH and SCNL, with the richness of the latter higher than the former (Figure 3.3A). Values of the diversity indices measured in Moora soils showed a very narrow range, from 0.983 and 0.987 (Figure 3.3B), with no effect of vegetation ($F_{6,22} = 1.624, P = 0.190$) and nitrogen treatments ($F_{1,22} = 2.609, P = 0.121$), and no interaction effect between the two ($F_{3,22} = 1.332, P = 0.291$). A similar trend was observed for evenness (Figure 3.3C), which had values of the Pielou’s index ranging between 0.778 and 0.801, and no significant effect of vegetation ($F_{6,22} = 0.935, P = 0.491$) and nitrogen treatment ($F_{1,22} = 0.409, P = 0.529$), nor significant interaction effects between the two ($F_{3,22} = 1.051, P = 0.391$).
Figure 3.3 Effects of treatments on OTU richness (A), Simpson’s index of diversity (B), and Pielou’s index of evenness (C). CNH = crop only with optimum N; CNL = crop only with low N; G = Gatton panic only; GCNH = Gatton panic and crop with optimum N; GCLH = Gatton panic and crop with low N; R = Rhodes grass only; RCNH = Rhodes grass and crop with optimum N; RCLN = Rhodes grass and crop with low N; S = Siratro only; SCNH = Siratro and crop with optimum N; SCNL = Siratro and crop with low N. Different letters indicate values that are significantly different ($P < 0.05$). Error bars represent ± one standard error ($n = 3$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns = not significant.
3.3.3. Microbial abundances

Bacterial and fungal communities were quantified by means of qPCR. The results are presented in Figure 3.4. Bacterial abundances ranged from $1.18 \times 10^7$ to $1.20 \times 10^8$ 16S rRNA gene copies g$^{-1}$ soil (Figure 3.4A). Differences between vegetation treatments and between nitrogen treatment were not significant ($F_{6,22} = 0.988, P = 0.45; F_{1,22} = 0.983, P = 0.332$ respectively). There was no statistically significant interaction between the effects of vegetation and nitrogen treatment on bacterial abundances ($F_{3,22} = 1.919, P = 0.156$).

Fungal abundances ranged from $4.66 \times 10^6$ to $5.20 \times 10^7$ 18S rRNA gene copies g$^{-1}$ soil (Figure 3.4B). No significant differences were observed in the abundances of fungal communities between vegetation and nitrogen treatments ($F_{6,22} = 1.046, P = 0.423; F_{1,22} = 0.033, P = 0.856$ respectively), while the interaction between the effects of the two treatments was marginally significant ($F_{3,22} = 3.095, P = 0.047$). However, when multiple comparisons were performed after ANOVA, the differences were not found significant.
3.3.4. Potential enzyme activity

The potential activities of β-glucosidase (BG), N-acetyl-glucosaminidase (NAG), and acid phosphatase (AP) measured at Moora experimental site are presented in Figure 3.5. A two-factor analysis of variance showed a significant effect of vegetation treatment on the potential activity of BG ($F_{6,22} = 6.020, P < 0.001$). The potential activity of soils in plots with crop only (CNH and CNL) was 40% higher...
than in plots with Gatton panic and crop (GCNH, GCNL) and 30% higher than soils treated with Rhodes grass and crop (RCNH, RCNL). Gatton panic only (G) and Siratro only (S) plots had higher BG activity than Gatton panic and crop plots (30 and 33%, respectively, Figure 3.5A). The effect of nitrogen treatment was not significant ($F_{1,22} = 0.111, P = 0.741$). There was no significant interaction between vegetation and nitrogen treatments ($F_{3,22} = 0.877, P = 0.468$). Similar results were found for the activity of NAG (Figure 3.5B), which had significantly different values across vegetation treatments ($F_{6,22} = 7.627, P < 0.001$). The enzyme activity in Gatton panic and crop plots was, in fact, between 30 and 42% lower than single crop and grass plots (i.e. CNH, CNL, G, R, S) and 41% lower than Siratro and crop plots (SCNH, SCNL). The effects of nitrogen treatment and of the interaction between vegetation and nitrogen were not significant ($F_{1,22} = 1.302, P = 0.266$; $F_{3,22} = 1.070, P = 0.382$ respectively). A significant effect of vegetation treatment was observed also for the potential activity of AP ($F_{1,6} = 7.952, P < 0.001$). Similarly to what observed for the other enzymes, Gatton panic and crop plots had lower AP activity when compared to single crop (35%) and grass plots (from 15 to 32%). Rhodes grass and crop plots showed activity 33% lower than crop only plots (Figure 3.5C). The effects of nitrogen treatment and of the interaction between vegetation and nitrogen were not significant ($F_{1,22} = 0.031, P = 0.862$; $F_{3,22} = 0.883, P = 0.465$ respectively).
Figure 3.5 Potential activity of β-glucosidase (A), N-acetyl-glucosaminidase (B), and acid phosphatase (C) across different treatments. CNH = crop only with optimum N; CNL = crop only with low N; G = Gatton panic only; GCNH = Gatton panic and crop with optimum N; GCLH = Gatton panic and crop with low N; R = Rhodes grass only; RCNH = Rhodes grass and crop with optimum N; RCNL = Rhodes grass and crop with low N; S = Siratro only; SCNH = Siratro and crop with optimum N; SCNL = Siratro and crop with low N. Error bars represent ± one standard error (n = 3). *** P < 0.001; ns = not significant.
3.3.5. Community respiration and substrate-induced respiration

Basal respiration rates at Moora experimental site (Figure 3.6A) ranged from 0.29 to 1.31 µg g\(^{-1}\) h\(^{-1}\) CO\(_2\)-C. The single effects of vegetation and nitrogen treatments were not significant (F\(_{2,22}\) = 1.414, P = 0.257; F\(_{5,22}\) = 2.760, P = 0.085, respectively) when analysed by a two-way ANOVA. A significant interaction of the effects of vegetation and nitrogen treatments was observed (F\(_{3,22}\) = 4.032, P = 0.019), but this was not confirmed by a Tukey’s pairwise comparison test. Overall soil respiration, measured by the sum of respiration responses of all 11 carbon substrates (Figure 3.6B) ranged from 0.32 to 3.08 µg g\(^{-1}\) h\(^{-1}\) CO\(_2\)-C. A two-factor ANOVA showed a significant effect of vegetation treatment on the total respiration response (F\(_{5,22}\) = 2.952, P = 0.034). Total soil respiration in the Gatton panic only plots (G) was 57% lower than in Siratro only plots (S). A significant effect of nitrogen treatment was also found (F\(_{2,22}\) = 8.429, P = 0.001). Both low and optimal nitrogen treatments had a negative impact on total respiration response, which was 32 and 48% lower in treated plots respectively than in plots left untreated (i.e. G, R, S). There was no significant interaction between vegetation and nitrogen treatments (F\(_{3,22}\) = 2.113, P = 0.127).
Figure 3.6 Mean respiration rates as measured for the control (i.e. no substrate added) (A) and sum of respiration responses to the 11 substrates (B) used in the MicroResp™ assay across different treatments. CNH = crop only with optimum N; CNL = crop only with low N; G = Gatton panic only; GCNH = Gatton panic and crop with optimum N; GCLH = Gatton panic and crop with low N; R = Rhodes grass only; RCNH = Rhodes grass and crop with optimum N; RCNL = Rhodes grass and crop with low N; S = Siratro only; SCNH = Siratro and crop with optimum N; SCNL = Siratro and crop with low N. Error bars represent ± one standard error (n = 3). * P < 0.05; ** P < 0.01; *** P < 0.001; ns = not significant.

3.3.6. Contribution of biotic and abiotic factors to soil functioning

The relationship between soil functions and microbial community factors and edaphic properties was investigated by using stepwise linear regressions. Results of the significant predictors identified for each function are presented in Table 3.6. None of the response variables showed correlation with OTUs. Hence, the
contribution of community composition to soil functioning was tested by including PCoA scores of the first two axes in the third group of variables.

There was no evidence for the three classes of biotic variables (i.e., diversity components, microbial abundances, and community composition) being predictors of any of the soil functions (Table 3.6). Bacterial diversity was initially found to be a significant predictor of potential BG activity and total substrate response, but when included in the final models, it was no longer significant ($P > 0.05$). Moreover, the model fit for the final model explaining the variation of BG resulted improved when bacterial diversity was excluded ($w_i = 0.601$ vs. $0.198$, data not shown). Edaphic properties were, hence, the only factors that significantly predicted the soil functions investigated in this study. Soil moisture content and the concentration of ammonia were identified as predictors of BG activity by the stepwise multiple regression procedure, while NAG activity was significantly predicted only by soil moisture. Potential AP activity was predicted by the concentrations of ammonia and phosphate. Bivariate correlations between soil moisture content and the activity of BG and NAG suggested positive linear relationships (Figure 3.7A, C), while $\text{NH}_4$ was correlated to BG and AP activity by negative linear relationships (Figure 3.7B, D). The correlation between the concentration of phosphate and AP activity also followed a negative relationship (Figure 3.7E). Basal respiration rates were best explained by the concentration of nitrate and total nitrogen. Within these, the most important predictor of basal respiration was total nitrogen ($\Delta \text{AICc} = 2.731$ vs. $2.292$, Table 3.7), which was correlated to the response variable by a positive linear relationship (Figure 3.7E). The bivariate correlation between $\text{NO}_3$ and basal respiration was, on the other hand, negative (Figure 3.7D). Bacterial diversity was
initially found to be the only significant predictor of total substrate response, but when individually regressed in the final model, it was no longer significant. Therefore, none of the variables investigated was found to be a significant predictor of total substrate responses. Nonlinearities between response and predictor variables were not detected by visual inspection for any of the soil functions (data not shown).
Table 3.6 Results of the multiple linear regressions constructed with each class of variables, for each soil function investigated.

<table>
<thead>
<tr>
<th>Function</th>
<th>1) Diversity components</th>
<th>2) Microbial abundances</th>
<th>3) Community composition</th>
<th>4) Edaphic factors</th>
<th>Final model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>P value</td>
<td>Var.</td>
<td>P value</td>
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<td>Fungi</td>
<td>0.877</td>
<td>PCo2</td>
</tr>
<tr>
<td></td>
<td>Diversity</td>
<td>0.175</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model P</td>
<td>0.577</td>
<td>0.974</td>
<td>0.361</td>
<td>0.100</td>
<td>0.151</td>
</tr>
<tr>
<td>Adj. R²</td>
<td>-0.033</td>
<td>-0.067</td>
<td>-0.003</td>
<td>0.139</td>
<td>0.151</td>
</tr>
<tr>
<td>AICc</td>
<td>184.00</td>
<td>178.87</td>
<td>181.34</td>
<td>186.02</td>
<td>174.85</td>
</tr>
<tr>
<td>AP</td>
<td>Richness</td>
<td>0.659</td>
<td>Bacteria</td>
<td>0.366</td>
<td>PCo1</td>
</tr>
<tr>
<td></td>
<td>Evenness</td>
<td>0.561</td>
<td>Fungi</td>
<td>0.880</td>
<td>PCo2</td>
</tr>
<tr>
<td></td>
<td>Diversity</td>
<td>0.351</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model P</td>
<td>0.698</td>
<td>0.652</td>
<td>0.815</td>
<td>0.073</td>
<td>0.025</td>
</tr>
<tr>
<td>Adj. R²</td>
<td>-0.052</td>
<td>-0.039</td>
<td>-0.054</td>
<td>0.209</td>
<td>0.169</td>
</tr>
<tr>
<td>AICc</td>
<td>244.40</td>
<td>236.04</td>
<td>242.93</td>
<td>243.07</td>
<td>235.30</td>
</tr>
<tr>
<td>Basal respiration</td>
<td>Richness</td>
<td>0.863</td>
<td>Bacteria</td>
<td>0.519</td>
<td>PCo1</td>
</tr>
<tr>
<td></td>
<td>Evenness</td>
<td>0.433</td>
<td>Fungi</td>
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<td>PCo2</td>
</tr>
<tr>
<td></td>
<td>Diversity</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model P</td>
<td>0.782</td>
<td>0.663</td>
<td>0.449</td>
<td>0.044</td>
<td>0.006</td>
</tr>
<tr>
<td>Adj. R²</td>
<td>-0.066</td>
<td>-0.040</td>
<td>-0.011</td>
<td>0.254</td>
<td>0.245</td>
</tr>
<tr>
<td>AICc</td>
<td>-88.15</td>
<td>-87.62</td>
<td>-91.33</td>
<td>-91.71</td>
<td>-100.73</td>
</tr>
<tr>
<td>Total resp. responses</td>
<td>Richness</td>
<td>0.351</td>
<td>Bacteria</td>
<td>0.892</td>
<td>PCo1</td>
</tr>
<tr>
<td></td>
<td>Evenness</td>
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<td>Fungi</td>
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<td>PCo2</td>
</tr>
<tr>
<td></td>
<td>Diversity</td>
<td>0.025</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model P</td>
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<td>0.763</td>
<td>0.186</td>
<td>0.855</td>
<td>0.835</td>
</tr>
<tr>
<td>Adj. R²</td>
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<td>0.048</td>
<td>-0.144</td>
<td>-0.031</td>
</tr>
<tr>
<td>AICc</td>
<td>-17.54</td>
<td>-12.60</td>
<td>-17.07</td>
<td>-1.84</td>
<td>-15.86</td>
</tr>
</tbody>
</table>
Table 3.7 Model fit for the final model that best explains variation of each function, for models in which the variables were dropped one by one, and for intercept-only models. Each row represents a model containing the variables for which the cells are filled. Models are ranked by declining explanatory power (Akaike weights \([w_i]\)).

<table>
<thead>
<tr>
<th>β-Glucosidase</th>
<th>Moisture</th>
<th>NH₄⁺</th>
<th>R²</th>
<th>AIC_c</th>
<th>Δ AIC_c</th>
<th>wᵢ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.267</td>
<td>168.319</td>
<td>0</td>
<td>0.516</td>
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<tr>
<td></td>
<td>Moisture</td>
<td></td>
<td>0.198</td>
<td>168.790</td>
<td>0.470</td>
<td>0.408</td>
</tr>
<tr>
<td></td>
<td>Moisture</td>
<td></td>
<td>0.0704</td>
<td>173.517</td>
<td>5.197</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>Moisture</td>
<td></td>
<td>0</td>
<td>173.573</td>
<td>5.253</td>
<td>0.037</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N-acetyl-glucosaminidase</th>
<th>Moisture</th>
<th>R²</th>
<th>AIC_c</th>
<th>Δ AIC_c</th>
<th>wᵢ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Moisture</td>
<td>0.141</td>
<td>183.291</td>
<td>0</td>
<td>0.800</td>
</tr>
<tr>
<td></td>
<td>Moisture</td>
<td>0</td>
<td>186.066</td>
<td>2.774</td>
<td>0.199</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Acid Phosphatase</th>
<th>NH₄⁺</th>
<th>PO₃⁻</th>
<th>R²</th>
<th>AIC_c</th>
<th>Δ AIC_c</th>
<th>wᵢ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.223</td>
<td>235.300</td>
<td>0</td>
<td>0.463</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.137</td>
<td>236.208</td>
<td>0.907</td>
<td>0.294</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.102</td>
<td>237.472</td>
<td>2.171</td>
<td>0.156</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>238.663</td>
<td>3.362</td>
<td>0.086</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Basal respiration</th>
<th>NO₃</th>
<th>Total N</th>
<th>R²</th>
<th>AIC_c</th>
<th>Δ AIC_c</th>
<th>wᵢ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.294</td>
<td>-100.736</td>
<td>0</td>
<td>0.619</td>
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</tr>
<tr>
<td></td>
<td>0.181</td>
<td>-98.443</td>
<td>2.292</td>
<td>0.197</td>
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</tr>
<tr>
<td></td>
<td>0.170</td>
<td>-98.004</td>
<td>2.731</td>
<td>0.158</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>-94.296</td>
<td>6.439</td>
<td>0.024</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.7 Bivariate correlations between significant variables in the final model and β-glucosidase (A, B), N-acetyl-glucosaminidase (C), acid phosphatase (D, E), and basal respiration (F, G).
3.4. Discussion

3.4.1. Effects of pasture cropping on bacterial community composition and structure

The purpose of this study was to investigate the nature of the relationship between microbial diversity and ecosystem functioning in a dryland pasture-cropping system. Nitrogen fertilisation was observed to have stronger effects on microbial communities than plant assemblages did. Bacterial community composition was in fact found to be significantly influenced by N amendments, while vegetation effects were not observed. The application of nitrogen to crops and pasture is a common practice in agricultural management and it has been widely reported to influence microbial community structure (e.g. Yao et al., 2014). In an experiment testing the effects of a gradient of N addition rates on soil microbial diversity and activity in grasslands, it was observed that rates similar to those used at Moora (5.25 g N m$^{-2}$ y$^{-1}$) changed bacterial community composition (Yao et al., 2014), supporting our findings. Also bacterial diversity was found to be affected by high rates of fertilisation. On the other hand, lower N addition rates, while still affecting the community composition, were not accompanied by a decrease in bacterial diversity. Since under higher N inputs, the soil pH was found to be 1 to 2 units lower than the control, the decrease in diversity was suggested to be associated to the decrease in soil pH (Yao et al., 2014).

In our study, we did not observe fertilisation effects on indices of diversity and evenness, but only on OTU richness and community composition, which was driven by soil pH. Soil pH is a primary factor affecting microbial species composition and function in soils (Fierer and Jackson, 2006, Lauber et al., 2008) including those in
Australia (Wakelin et al., 2008). Despite that these study soils had a small gradient of pH (6.71 to 7.33), this was found to drive differences in community composition. Contrarily to the work of Yao et al. (2014), we did not observe significant effects on indices of diversity and evenness but only on OTU richness. Increases of N availability associated to long-term fertilisation did not affect bacterial diversity and richness of a grassland soil and an agricultural field soil (Ramirez et al., 2010).

The different vegetation assemblages tested in this pasture-cropping experiment did not produce significant differences in bacterial community composition. Soil bacterial communities have been shown to vary between crop species (Wieland et al., 2001, Garbeva et al., 2004). In a study that compared continuous cotton paddock with an integrated crop and perennial grass pasture system, fatty acid methyl ester analysis revealed differences in microbial composition between perennial pastures compared to continuous crop (Acosta-Martinez et al., 2004). Bacterial community composition in the rhizosphere is primarily affected by plant factors, but it is also affected by edaphic factors such as soil type, nutrient availability and pH (Marschner et al., 2004). In an experiment investigating the effects of fertilisation on bacterial community structure associated to barley and cucumber grown in two different soil types, it was found that the bacterial community structure was soil-specific, did not differ between the two plant species, and it was affected by fertilisation (Marschner et al., 2004). It is therefore possible that in our study soil factors were more important for determining the bacterial community composition than plant effects, specifically pH was a significant driver of bacterial community composition. Soil pH can also affect the bacterial community structure indirectly by influencing nutrient availability and root exudates amount and composition (Marschner et al., 2004).
Despite no direct effect of the plant species was observed, it is possible that pasture-cropping treatments influenced the bacterial composition via indirect effects of nitrogen treatment and soil pH on vegetation.

Below-ground diversity has been observed to decrease with N fertilisation in pasture, as the quantity and quality of litter entering the soil food web changes (Donnison et al., 2000). In grassland soils, both the bacterial and fungal communities may experience a reduction in diversity in response to N fertilizer (Kennedy et al., 2004, 2005). In this study, no treatment effects were observed on bacterial diversity and evenness, while bacterial richness was influenced by the single effects of pasture-cropping treatments and nitrogen treatments, and by the interaction between them. The lack of significant differences in diversity and evenness indices across plots suggests that pasture-cropping treatments affected the number of bacterial phylotypes rather than the relative abundances of phylotypes.

3.4.2. Drivers of soil functioning in a pasture-cropping system

This study investigated the relationship between microbial diversity components and soil processes catalysed by soil microbes in an agricultural system subjected to pasture-cropping treatments. Based on the knowledge that agricultural management has important effects on microbial community structure (Garbeva et al., 2004, Bünemann et al., 2006), it was expected to observe changes in soil biotic and abiotic properties in order to investigate their relations to soil functioning. In this study, soil pH and concentration of ammonia responded to pasture-cropping treatments. While pH did not result particularly important in predicting soil functioning, the concentration of ammonia was a significant predictor of the potential activity of β-glucosidase and acid phosphatase. On the other side, soil pH appeared to be a
primary driver of bacterial community composition. Hence, both microbial community and functional responses to treatment were observed, but nonetheless no statistical correlation between microbial properties and soil functioning was found, indicating that these responses were independent from each other. The functioning of soil in this pasture-cropping system seemed to be driven only by edaphic properties, in particular by soil moisture content and the concentration of ammonia, nitrate and total nitrogen. This can be due to the fact that pasture-cropping treatments did not produce strong changes across plots in biotic properties. In fact the differences in diversity and evenness were not significant, and a small gradient of OTU richness was found. Also the effects on enzyme activity, soil respiration and utilisation of carbon substrates were not particularly prominent. It is therefore difficult to detect any relationship between bacterial community structure and soil functioning. Moreover, given the ubiquitous distribution of the ability of degrading C in heterotrophs, it is perhaps not surprising that there was no discernible relationship between microbial community structure and composition and functions involved in decomposition, as observed for soil respiration across a land-use gradient from conventional crop agriculture to intact forest (Levine et al., 2011). The lack of relationship between bacterial diversity and soil respiration has been documented for other systems and soil types (Balser and Firestone, 2005, Wertz et al., 2006).

Another possible explanation for the lack of observation of any correlation between microbial parameters and functions could be that agricultural practices may have disrupted the biodiversity-ecosystem functioning relationship in soils. In fact, in agricultural systems, a range of stresses can be imposed on soils, including tillage, cultivation, fertilisers, which may damage soil physical and biological properties and
compromise the ability of the soil to function. Other than directly affecting soil belowground diversity (Culman et al., 2010, Postma-Blaauw et al., 2010), these stresses might have produced at the Moora experimental site a homogenisation of the soil environment, resulting in a simplification of species interactions and leading to a loss of a direct link between microbial communities and soil functions. It has been recognised that the shape and direction of the biodiversity-functioning relationship can depend critically on the environmental context (Cardinale et al., 2000, Fridley, 2002, Tylianakis et al., 2008). Theoretical studies suggest that spatial heterogeneity in the availability of soil resources and disturbance can influence the nature of the relationship between diversity and productivity in plant systems (Cardinale et al., 2000, Maestre and Reynolds, 2007). Complementarity effects, whereby resource partitioning among species leads to increased resource use and therefore to increased functioning of the system, would be more evident in more heterogeneous habitats. This has been demonstrated for the relationship between plant diversity and plant biomass in grasslands, which became steeper with higher heterogeneity (variation) of soil properties, without direct effects of heterogeneity on diversity (Tylianakis et al., 2008). Because soil properties did not vary greatly across treatments, the failure in observing any strong relationship between microbial components and functioning might be attributed to the homogenisation of the soil environment by the pasture-cropping management. Because this has only been proven for plant systems, further research is necessary to confirm the validity of this hypothesis for soil microbial communities across different agricultural managements.

The lack of any observable link between bacterial community structure and the soil functional properties investigated also suggests that other unmeasured biotic factors
were contributing to the functioning. This study only focused on bacterial diversity, while fungi have been reported to be more resistant than bacteria to drought and heat (Schimel et al., 2007, Acosta-Martínez et al., 2014). Because the moisture content of the soils at Moora experimental site was very low (< 2%), it is possible that the fungal communities were better adapted to the dry conditions than the bacterial communities and therefore have a stronger role in soil functioning. Soil moisture, in fact, directly influences the physiological status of bacteria and it may limit their ability to carry out functions such as decomposition. Moisture limitation can also affect bacteria through starvation but also induced osmotic stress and resource competition thus applying a strong selective pressure on the structure and functioning of bacterial communities (Griffiths et al., 2003).

3.5. Conclusions and future directions

The effects of management on soil microbiology and functionality in pasture-cropping systems is not well documented. The pasture-cropping treatments (combination of different perennial grasses with crop, under different nitrogen regimes) applied at the Moora experimental site influenced bacterial community composition and richness mainly through nitrogen amendments, though without creating strong differences in the soil functionality. The effects of nitrogen amendments were more pronounced than the effects of plant assemblages on the belowground communities. This suggests that fertilisation can have important impacts on soil through the modification of both the soil nutrient levels and the composition and functionality of the soil microbial community. No relationship between the soil biotic parameters examined and soil functioning was detected. The
main predictors of soil functioning were abiotic factors. This suggests that the soil environment played a stronger role in influencing the functional properties than the biotic component in these soils. Therefore, agricultural management influenced the soil functioning by modification of the soil properties. Further research is needed to confirm this observation across different management and soil types. The extreme complexity of soil biodiversity needs to be considered in all its components for a complete understanding of soil functioning. Because microbial communities are crucial to agricultural productivity, pasture-cropping management needs to be oriented towards practices that improve the functioning of soil microbial communities.
CHAPTER 4

Microbial diversity and soil functioning in a forest ecosystem
4.1. Introduction

The study of biodiversity-ecosystem function relationships (BEF) in microbial communities has been mainly addressed by means of controlled laboratory experiments (*e. g.* Bell *et al.*, 2005, Jiang, 2007, Philippot *et al.*, 2008). In these studies, bacterial diversity was directly manipulated in order to analyse the effects of species loss on the performance of the ecosystem function of interest. Although microbial microcosm studies have been useful in shedding some light on the biodiversity-ecosystem functioning relationship in microbial communities (Petchey *et al.*, 2002), they have been criticised because artificially constructed or manipulated communities are considered too simplified to be realistic representations of what occurs in nature (Jessup *et al.*, 2004). Because most microbes are unculturable (*Sait et al.*, 2002, *Joseph et al.*, 2003), it is generally impossible to experimentally assemble communities from a set of culturable species that accurately reflects communities from natural ecosystems. Experiments using culture-independent techniques to manipulate (*i. e.* dilution-to-extinction or fumigation approach) (Griffiths *et al.*, 2004, Girvan *et al.*, 2005, Wertz *et al.*, 2006) or describe microbial diversity and simultaneously measure ecosystem process rates *in situ* (Cavigelli and Robertson, 2000, Griffiths *et al.*, 2001a) represent a valid alternative to the culture-dependent approach. The advantage of this approach is that the results apply to whole microbial communities and not only to the subsets that can be cultured. The general disadvantage of microbial diversity manipulations is that biodiversity effects tend to be masked by differences among species in their susceptibility to elimination during the biodiversity manipulation procedure (Bell *et al.*, 2009).
Comparative studies that used natural differences in soil nitrifier communities found that the identity of the species in the soil affected rates of ammonia oxidation, and therefore concluded that there is potential for important effects of nitrifier community composition and diversity on nitrification rates (Cavigelli and Robertson, 2000). Another study investigating the BEF in soils with different levels of biodiversity did not find any link between microbial diversity and substrate utilization (Griffiths et al., 2001a). Because of the paucity of field studies on the BEF, the effects of microbial diversity on soil ecosystem functioning are still not clear, particularly when considering the influence of land use and land management practices. Land management practices can significantly alter soil physical, chemical, and biological properties, which in turn can have profound effects on many ecosystem functions, as observed in forest ecosystems (Waldrop et al., 2003, Purahong et al., 2014). In forest soils, the growth, activity and structure of the soil microbial community are affected by abiotic and biotic factors including quality and quantity of organic matter input, nutrient availability and physical disturbance (Leckie et al., 2004, Hannam et al., 2006, He et al., 2006). These factors may in turn be influenced by land-use change (e.g. natural forest vs. managed plantation) and management techniques (Zheng et al., 2005, He et al., 2006, Burton et al., 2010, Gao et al., 2014).

The purpose of this study was to investigate the nature of the BEF in field soils under realistic environmental conditions, and how this relationship is influenced by land-management practices and environmental heterogeneity in a forest plantation experiment. It is hypothesised that the BEF is stronger in forest ecosystems compared to agricultural systems where a higher management intensity may disrupt the relationship between microbial communities and soil functioning.
Soils were chosen from two sources: a forest management experiment, where a eucalypt plantation was subjected to different irrigation and fertilisation treatments, and along a vegetation transect in a natural woodland. This gave a wide range of environmental characteristics varying from management practices expected to influence diversity by changing edaphic properties, to changes in plant diversity, and thus a range of expected differences in soil microbial diversity. In fact, microbial communities may experience a reduction in diversity in response to nitrogen fertiliser (Kennedy et al., 2004, 2005), while irrigation is known to have positive effects on microbial biomass by increasing soil moisture (Litton et al., 2003, Samuelson et al., 2009). The link between diversity and soil functioning was investigated by means of a model-based approach evaluating the contribution of microbial and edaphic factors to functions involved in carbon and nutrient cycling of naturally diverse communities.

4.2. Materials and Methods

4.2.1. Sites description and soil sampling

Soils came from two sites: the Hawkesbury Forest Experiment (HFE) site (University of Western Sydney, Richmond, NSW, Australia) and the adjacent Cumberland Plain woodland. The HFE consists of four treatments: control (C), irrigation (I), fertilisation (F) and irrigation and fertilisation (IF), replicated in four plots (38.5 × 41.6 m) in a randomized complete block design. The HFE was established in April 2007, when each plot was planted with 160 *Eucalyptus saligna* in ten rows of 16 trees. Each tree, across all treatments, was supplied with 50 g
diammonium phosphate starter blend (N 15.3%, P 8.0%, K 16.0%, S 7.7% and Ca 0.3%) per plant to facilitate tree establishment. The C treatment received no additional fertiliser or water (besides ambient rainfall). The F and IF treatments were fertilised for the first time in January 2008 with a solid N fertiliser (N 20.6%, P 3.0%, K 7.5%, S 3.8%, Ca 4.4%) at a rate of 25 kg N ha\(^{-1}\) year\(^{-1}\). Solid N fertiliser (N 21.6%, P 8.1%, K 12.0%, S 0.6%), at a rate of 150 kg N ha\(^{-1}\) year\(^{-1}\), was applied uniformly to the F treatment beginning in October 2008. In the I treatment, water was supplied at a rate of 15 mm every 4 days from September to April, and 7–10 mm every 4 days from April to September. Therefore, the irrigated plots received about 1000 mm year\(^{-1}\) plus the ambient rainfall. The IF treatment received liquid fertilizer at a rate of 150 kg N ha\(^{-1}\) year\(^{-1}\), beginning in October 2008. The total amount of water added into the IF treatment was the same to the I treatment.

In addition to the HFE plots, eight sites were sampled in the adjacent natural woodland along a spatial transect circa 1.72 km, following a vegetation gradient, with the hypothesis that different vegetation assemblages and local environmental conditions would be associated with different levels of soil microbial diversity. These samples were collected in order to extend the diversity range of the soil microbial communities sampled in the HFE site. Location and dominant vegetation characteristics of these sites (NW) are listed in Table 4.1.

Soil samples were collected from the 16 plots of the HFE experiment and from the eight sites in the adjacent forest in March 2014, for a total of 24 samples. Eight soil cores (2 cm in diameter and 10 cm deep) were collected within ten meters from the edge of each of the four replicate HFE plots, between trees in multiple rows approximately one meter from the base of trees, and from the eight natural sites one
meter around trees and peculiar vegetation. The cores were then pooled and homogenized into a composite sample for each plot or site, sieved through a 2 mm mesh, and stored in plastic bags at 4 °C in the dark until analyses.

**Table 4.1.** Location and vegetation description of natural forest sites.

<table>
<thead>
<tr>
<th>Site ID</th>
<th>Coordinates</th>
<th>Dominant Vegetation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NW 1</td>
<td>S 33°36.642, E 150°43.449</td>
<td><em>Eucalyptus floribunda</em>; <em>Melaleuca</em> spp., vines, brambles</td>
</tr>
<tr>
<td>NW 2</td>
<td>S 33°36.714, E 150°43.358</td>
<td><em>Angophora</em> spp.</td>
</tr>
<tr>
<td>NW 3</td>
<td>S 33°36.808, E 150°43.366</td>
<td><em>Melaleuca decora</em></td>
</tr>
<tr>
<td>NW 4</td>
<td>S 33°36.913, E 150°43.464</td>
<td><em>E. tereticornis</em></td>
</tr>
<tr>
<td>NW 5</td>
<td>S 33°36.868, E 150°43.719</td>
<td><em>Corymbia</em> spp.</td>
</tr>
<tr>
<td>NW 6</td>
<td>S 33°37.015, E 150°43.938</td>
<td><em>E. tereticornis</em>; <em>Melaleuca</em> decora*; grassy understory</td>
</tr>
<tr>
<td>NW 7</td>
<td>S 33°37.258, E 150°44.259</td>
<td><em>E. tereticornis</em></td>
</tr>
<tr>
<td>NW 8</td>
<td>S 33°37.291, E 150°44.049</td>
<td>Wetland edge; open grassy vegetation</td>
</tr>
</tbody>
</table>

**4.2.2. Determination of relative bacterial diversity and richness and microbial abundances**

The total genomic DNA was isolated following the procedure described in section 3.2.2. Illumina MiSeq 16S amplicon sequencing (Illumina Inc. San Diego, CA, USA) was carried out as described in section 3.2.2. Data analysis was performed
using the ‘Quantitative Insights Into Microbial Ecology’ (QIIME v 1.8.0) software package (Caporaso et al., 2010) following the procedure described in section 3.2.2. Bacterial and fungal abundances were quantified by means of qPCR as described in section 3.2.3. The bacterial and fungal gene copies number was used to calculate the fungal:bacterial ratio, used as a microbial community variable in the multiple regressions model (see section 4.2.5).

4.2.3. Functional measurements: community respiration and potential enzyme activity

Basal respiration and total substrate respiration responses were measured by means of the MicroResp™ method as described in section 3.2.4.2. Soils were placed into the deep-well plates on the collection day and the assay was carried out at field moisture after two days of incubation in the dark at room temperature. The calculation of the rate of CO₂ respiration per gram of soil by means of the formula reported in section 3.2.4.2. accounts for the differences in field moisture across soil samples.

The potential activities of β-glucosidase (BG), N-acetyl-glucosaminidase (NAG), and acid phosphatase (AP) were determined using fluorimetric assays following the procedure described in section 3.2.4.1. The concentration of synthetic substrates used in these assays was optimised for the soils under investigation (see Appendix D). The assays were carried out the day after the soil samples were collected.
4.2.4. Soil physicochemical analyses

The soil physicochemical analyses (exchangeable nitrate, extractable phosphate, total carbon and nitrogen, soil pH and soil moisture content) were carried out as described in section 3.2.5.

4.2.5. Statistical methods

All the statistical analyses were carried out using the software R 3.1.1 (R Core Development Team 2014). Bacterial community composition (relative OTU abundance data) was visualised using principal coordinates analysis (PCoA, Gower, 1966) based on a Bray-Curtis distance matrix. Permutational Multivariate Analysis of Variance (PerMANOVA, Anderson, 2001) was conducted to assess the significance of treatment effects. Community data were also explored using Bray-Curtis distance-based redundancy analysis (dbRDA, Legendre and Anderson, 1999) to reveal relationships between bacterial communities and the soil physico-chemical properties measured. The edaphic variables that significantly influenced the bacterial communities were identified by a backward stepwise selection method using permutation tests (Blanchet et al., 2008). All the bacterial community data analyses were performed using the R package ‘vegan’ (Oksanen et al., 2014)

The data of edaphic properties, diversity indices (i.e. OTU richness, Simpson’s diversity index, Pielou’s index of evenness), microbial abundances, and community respiration (i.e. basal respiration and sum of respiration responses) were analysed separately using a one-way ANOVA with management as predictor. The dependent variables were tested for normality and homoscedasticity by using the Shapiro-Wilk’s test and the Bartlett’s test, respectively. Bacterial and fungal abundances, pH,
moisture, total C and N data were log-transformed in order to meet the homoscedasticity assumption of ANOVA. Where significant differences for the main effect were observed ($P < 0.05$), a Tukey’s pairwise comparison test was also performed.

The enzyme activity data were analysed by means of a Kruskall-Wallis rank sum test (Hollander and Wolfe, 1979). Where significant differences between treatments ($P < 0.05$) were observed, a multiple comparison test was performed (Siegel and Castellan). Community level physiological profiles (CLPP) were generated by subtracting the water response from each individual substrate response. dbRDA followed by a backward stepwise selection method by permutation tests, was used on CLPP data to test the statistical significance of each the edaphic factors. A permutation test was then used to test the significance of the selected model.

Soil functioning was analysed by means of stepwise multiple regressions examining the explanatory power of microbial community variables and edaphic factors, following the approach described in section 3.2.6.

The influence of forest management on soil functions was evaluated by means of structural equation modelling (Malaeb et al., 2000, Grace et al., 2007), analysing the effects of management practices (i.e. irrigation and fertilisation) and land use (managed forest vs. unmanaged natural woodland) on the significant predictors of each function. The same conceptual model of the relationship between response variables, predictors, and management practices (Figure 4.1) was applied to each function. The model fit was assessed by means of a chi-squared test and the comparative fit index (CFI). Data supported the models when $P > 0.05$ and CFI > 0.95. The strength of the modelled relationships between variables were quantified
by the estimated coefficients and tested for significance using z statistics. The analyses were performed using the R package ‘lavaan’ (Rosseel, 2012).

![Figure 4.1](image)

**Figure 4.1** Illustration of the relationship tested by structural equation modelling. The model represents direct, causal relationships among variables.

### 4.3. Results

#### 4.3.1. Soil properties

The soil physico-chemical properties are reported in Table 4.2. Forest management practices significantly influenced soil pH ($F_{4,18} = 8.850, P < 0.001$), which was higher in irrigation (I) and irrigation + fertilisation (IF) treatments, compared to control (C). Despite the lowest soil pH in the fertilisation treatment (F), it was not significantly different from C. No effects of land use (managed forest vs. natural woodland) were observed on soil pH. Soil moisture was three times higher in the irrigated treatments (I and IF) and in the natural woodland samples (NW) than in C and F ($F_{4,18} = 5.447, P = 0.004$). Total carbon and nitrogen were not significantly different across different management treatments, but significantly higher values were observed in the NW soils ($F_{4,18} = 4.487, P = 0.010$ for total carbon and $F_{4,18} =$
3.920, \( P = 0.018 \) for total nitrogen). The concentration of phosphate (\( \text{PO}_4^{3-} \)) in soils under F and IF treatment was over four and three times higher respectively than in control soils (\( F_{4,18} = 80.496, \ P < 0.001 \)). No significant effect of land use was observed on soil phosphate. Soil nitrate (\( \text{NO}_3^- \)) was not found to be significantly different across treatments (\( F_{4,18} = 2.135, \ P = 0.120 \)).

**Table 4.3** Mean values of soil pH, soil moisture content, total carbon, total nitrogen, phosphate concentrations (\( \text{PO}_4^{3-} \)), ammonium concentrations (\( \text{NH}_4^+ \)), nitrate concentrations (\( \text{NO}_3^- \)) across different forest-management treatments. IF = irrigation + fertilisation; C = control; F = fertilisation; I = irrigation; NW = natural woodland. Different letters indicate values that are significantly different (\( P < 0.05 \)). Values in parenthesis represent ± one standard error (n=8 for NW, n=4 for the others).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH</th>
<th>Moisture (% soil weight)</th>
<th>Total C (g kg(^{-1}))</th>
<th>Total N (g kg(^{-1}))</th>
<th>( \text{PO}_4^{3-} ) (mg kg(^{-1}))</th>
<th>( \text{NO}_3^- ) (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>7.19(^b)</td>
<td>3.22(^{ab})</td>
<td>10.92(^{ab})</td>
<td>0.78(^{ab})</td>
<td>8.15(^{cd})</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>(0.56)</td>
<td>(0.32)</td>
<td>(2.09)</td>
<td>(0.14)</td>
<td>(1.10)</td>
<td>(0.01)</td>
</tr>
<tr>
<td>F</td>
<td>6.09(^b)</td>
<td>2.96(^b)</td>
<td>10.46(^b)</td>
<td>0.67(^b)</td>
<td>45.62(^a)</td>
<td>3.06</td>
</tr>
<tr>
<td></td>
<td>(0.15)</td>
<td>(0.26)</td>
<td>(0.27)</td>
<td>(0.03)</td>
<td>(3.85)</td>
<td>(0.66)</td>
</tr>
<tr>
<td>I</td>
<td>9.18(^{ac})</td>
<td>9.11(^a)</td>
<td>10.34(^{b})</td>
<td>0.76(^{ab})</td>
<td>15.07(^{c})</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>(0.17)</td>
<td>(1.50)</td>
<td>(1.59)</td>
<td>(0.04)</td>
<td>(1.82)</td>
<td>(0.47)</td>
</tr>
<tr>
<td>IF</td>
<td>8.60(^c)</td>
<td>9.53(^a)</td>
<td>9.90(^{b})</td>
<td>0.72(^{ab})</td>
<td>33.72(^{b})</td>
<td>4.66</td>
</tr>
<tr>
<td></td>
<td>(0.18)</td>
<td>(0.98)</td>
<td>(0.11)</td>
<td>(0.008)</td>
<td>(2.43)</td>
<td>(2.14)</td>
</tr>
<tr>
<td>NW</td>
<td>7.25(^{bc})</td>
<td>9.80(^a)</td>
<td>27.14(^{a})</td>
<td>1.93(^a)</td>
<td>4.21(^{d})</td>
<td>1.82</td>
</tr>
<tr>
<td></td>
<td>(0.46)</td>
<td>(2.59)</td>
<td>(6.57)</td>
<td>(0.57)</td>
<td>(0.66)</td>
<td>(0.49)</td>
</tr>
</tbody>
</table>

**4.3.2. Bacterial community composition, structure and diversity**

Between 17 907 and 52 128 unique sequences were obtained after quality filtering, with an average of 33 919 sequences per sample. These were subsequently clustered into 52 128 OTUs.
The bacterial community composition as revealed by the PCoA of sequencing data (Figure 4.2A) was influenced by forest management treatments. I and IF samples were separated from the other samples along PCo axis 1 (60.63% of the variation explained), while F samples had a distinct microbial assemblage compared to those of other treatments on the PCo axis 2 (18.37% of the variation explained). PerMANOVA analysis confirmed the significant dissimilarity in bacterial communities between treatments ($P = 0.001$). Relative abundance data at phylum level showed that change in community composition was mainly driven by dominance of *Proteobacteria* at the expense of *Acidobacteria* and *Actinobacteria* in the irrigated treatments (Figure 4.2B).
Figure 4.2 Ordination plot of the first two axes produced by principal coordinates analyses of Bray–Curtis distances of bacterial community composition (A), and relative abundance of phyla based on amplicon 16S rRNA gene data (B). NW = natural woodland; C = control; F = fertilisation; I = irrigation; IF = irrigation + fertilisation.

To identify the main environmental factors associated with bacterial community composition, dbRDA was carried out with soil physico-chemical parameters as
environmental variables and sequencing data (Figure 4.3). The results show that pH, soil moisture content and extractable phosphates (P) were significantly associated with bacterial community composition ($P = 0.005$, $0.015$, and $0.005$ respectively). The bacterial communities of I and IF treatments were associated with higher values of soil pH and moisture, while phosphate positively influenced the communities of the F treatment.

**Figure 4.3** Ordination plot of the first two axes produced by redundancy analysis based on Bray–Curtis distances. Effects of edaphic variables on bacterial community composition, based on amplicon 16S rRNA gene data. Numbers in parenthesis are the percentage of variation explained by each of the plotted axis. NW = natural woodland; C = control; F = fertilisation; I = irrigation; IF = irrigation + fertilisation.

OTU richness ranged from average values of $324 \pm 9.76$ (F) to $483 \pm 8.26$ (IF) OTUs (Figure 4.4A). Plots subjected to irrigation (I) and to irrigation + fertilisation (IF) treatments had higher richness compared to the other HFE treatments and the natural woodland soils ($F_{4,18} = 26.85$, $P < 0.001$). A similar trend was observed in the other
diversity indices analysed. The values of the Simpson’s diversity index (Figure 4.4B) ranged from an average of 0.96 ± 0.002 (F) to 0.98 ± 0.0002 (I), with I and IF treatments featured by a significantly higher diversity (F_{4,18} = 17.7, P < 0.001) compared to the remaining treatments.

The evenness of the bacterial community, measured by Pielou’s index of evenness (Figure 4.4C), was significantly higher in I and IF plots (F_{4,18} = 26.36, P < 0.001), and it ranged from average values of 0.70 ± 0.007 (F) to 0.79 ± 0.001 (I).
Figure 4.4 Effects of forest management treatments on OTU richness (A), Simpson’s index of diversity (B), and Pielou’s index of evenness (C) calculated on sequencing data. NW = natural woodland; C = control; F = fertilisation; I = irrigation; IF = irrigation + fertilisation. Different letters indicate values that are significantly different ($P < 0.001$). Error bars indicate ± one standard error (n=8 for NW, n=4 for the others).
4.3.3. Microbial abundances

Bacterial and fungal communities were quantified by means of qPCR. The results are presented in Figure 4.5. The bacterial abundances were significantly affected by treatment ($F_{4,19} = 8.427, P < 0.001$), being lower in the fertilised soil ($2.49 \times 10^6$ copies g$^{-1}$ soil) compared to IF ($7.36 \times 10^6$ copies g$^{-1}$ soil), I ($9.86 \times 10^6$ g$^{-1}$ soil), and NW ($1.58 \times 10^7$ g$^{-1}$ soil) soils (Figure 4.5A).

Significant differences were also observed across treatments in fungal abundances ($F_{4,19} = 8.351, P < 0.001$), which had average values ranging from $5.22 \times 10^5$ g$^{-1}$ soil in the IF treatment, to $3.08 \times 10^6$ g$^{-1}$ soil in the NW soils (Figure 4.5B).

**Figure 4.5** Bacterial (A) and fungal (B) abundances across different treatments. NW = natural woodland; C = control; F = fertilisation; I = irrigation; IF = irrigation + fertilisation. Error bars indicate ± one standard error (n=8 for NW, n=4 for the others). Different letters above the bars indicate a significant difference ($P < 0.001$).
4.3.4. Potential enzyme activity

The potential enzyme activity results are shown in Figure 4.6. An overall higher activity was observed in the irrigated treatments. A Kruskall-Wallis test showed that there was a statistically significant difference in the activity of β-glucosidase between the different treatments ($\chi^2(4) = 9.930, P = 0.041$; Figure 4.6A). The multiple comparisons after Kruskall-Wallis test were not found to be significant. Significant differences were observed in the potential activity of N-acetyl-glucosaminidase ($\chi^2(4) = 10.477, P = 0.033$; Figure 4.6B), where the irrigated soils (I) had the highest potential activity (89.83 ± 4.27 nmol g$^{-1}$ soil h$^{-1}$) and the fertilised soils (F) the lowest (68.17 ± 3.06 nmol g$^{-1}$ soil h$^{-1}$). The activity of acid phosphatase was higher in I (308.92± 10.66 nmol g$^{-1}$ soil h$^{-1}$) and IF soils (306.92± 8.33 nmol g$^{-1}$ soil h$^{-1}$) compared to F soils (200.58 ± 6.61 nmol g$^{-1}$ soil h$^{-1}$), with significant differences observed ($\chi^2(4) = 17.617, P = 0.001$; Figure 4.6C).
Figure 4.6 Potential activity of β-glucosidase (A), N-acetyl-glucosaminidase (B), and acid phosphatase (C) in the different soil treatments. NW = natural woodland; C = control; F = fertilisation; I = irrigation; IF = irrigation + fertilisation. Different letters indicate values that are statistically different ($P < 0.05$). Error bars indicate ± one standard error (n=8 for NW, n=4 for the others).
4.3.5. Community respiration and substrate-induced respiration

Overall substrate induced respiration, measured by the sum of respiration responses of all 11 carbon substrates (Figure 4.7), was significantly higher in the irrigation and fertilisation treatment (9.05 ± 1.60 µg g⁻¹ h⁻¹ CO₂-C) and in the natural woodland (7.07 ± 0.94 µg g⁻¹ h⁻¹ CO₂-C), while the lowest respiration rates were observed in the control (1.60 ± 0.42 µg g⁻¹ h⁻¹ CO₂-C) and fertilised soils (1.06 ± 0.44 µg g⁻¹ h⁻¹ CO₂-C) (F₄,₁₉ = 11; P < 0.001).

![Figure 4.7](image)

**Figure 4.7** Sum of respiration responses to the 11 substrates used in the MicroResp™ assay in the different treatments. IF = irrigation + fertilisation; C = control; F = fertilisation; I = irrigation; NW = natural woodland. Different letters indicate values that are statistically different (P < 0.001). Error bars indicate ± one standard error (n=8 for NW, n=4 for the others).

Similar results were found for the basal respiration (Figure 4.8), measured as the respiration rates of the control wells of the MicroResp™ assay (i.e. without the addition of substrates). In fact, the respiration rates of IF and NW soils (1.48 ± 0.08 and 1.43 ± 0.09 µg g⁻¹ h⁻¹ CO₂-C respectively) were significantly higher than those
measured in C and F treatments (0.88 ± 0.08 and 0.97 ± 0.15 µg g⁻¹ h⁻¹ CO₂-C respectively; F₄,₁₉ = 7.103, P = 0.001).

**Figure 4.8** Mean respiration rates as measured for the control (i.e. no substrate added) in the MicroResp™ assay in the different treatments. IF = irrigation + fertilisation; C = control; F = fertilisation; I = irrigation; NW = natural woodland. Different letters indicate values that are statistically different (P < 0.001). Error bars indicate ± one standard error (n=8 for NW, n=4 for the others).

To identify the main factors affecting the carbon source utilization patterns, dbRDA was carried out on the substrate respiration responses with soil physico-chemical parameters as environmental predictors (Figure 4.9). Forest management treatments had a significant effect on the CLPP, which was mainly driven by total carbon (C, P = 0.005), as determined by stepwise selection by permutation test.
4.3.6. Relationship between microbial communities and soil functions

The relationship between soil functions and the microbial community properties was evaluated by estimating the independent contribution of biotic and abiotic factors to the potential enzymatic activities and soil respiration using a model-based approach. Results of the significant predictors of each soil function identified via the step-wise procedure are presented in Table 4.3. None of the response variables showed correlation with individual OTUs. Thus, the contribution of community composition to soil functioning was tested by including PCoA scores of the first two axes in the third group of variables. All soil functional responses were predicted by both biotic
and abiotic components, except N-acetyl-glucosaminidase activity, which was best predicted by edaphic factors only (i.e., pH and soil moisture). Several variables were identified during the initial step-wise procedure as significant predictors of each function, but they were no longer significant when combined in the final model. This is probably due to a covariance of these variables with others that were better predictors of the function.

Within the biotic factors, the community composition class had the highest explanatory power (Adjusted $R^2$) for the potential enzyme activities compared to the other classes of variables (Table 4.3), while for respiration functions microbial abundances were more powerful. Edaphic factors had higher explanatory power than biotic properties on for BG and NAG activities and basal respiration. The potential activity of BG was best explained by OTU richness and soil moisture content (Table 4.4), being richness the most important ($\Delta$AICc = 6.069; Table 4.4). Variables belonging the edaphic factors class were found to explain the variance of NAG, where the most important predictor was soil pH ($\Delta$AICc = 8.316; Table 4.4). AP activity was best predicted by OTU richness and soil moisture. When the importance of the predictors was compared, richness was found to be the most important ($\Delta$AICc = 30.281; Table 4.4).

None of the diversity components and community structure explained basal respiration (Table 4.4), which was best explained by bacterial abundance and the concentration of nitrate. Bacterial abundance was found to be the most important predictor of basal respiration ($\Delta$AICc = 11.090; Table 4.4). Total substrate response was explained by OTU richness and total N, which had similar importance in predicting this response ($\Delta$AICc = 6.832 and 6.863; Table 4.4).
Table 4.3 Results of the multiple linear regressions constructed with each class of variables, for each ecosystem function investigated.

<table>
<thead>
<tr>
<th>Function</th>
<th>1) Diversity components</th>
<th>2) Microbial abundances</th>
<th>3) Community composition</th>
<th>4) Edaphic factors</th>
<th>Final model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Var.</td>
<td>P value</td>
<td>Var.</td>
<td>P value</td>
<td>Var.</td>
</tr>
<tr>
<td>BG</td>
<td>Richness &lt;0.001</td>
<td>Bacteria 0.554</td>
<td>PCo 1 &lt;0.001</td>
<td>pH 0.004</td>
<td>Rich. &lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Evenness 0.409</td>
<td>Fungi 0.374</td>
<td>PCo 2 0.295</td>
<td>NO₃ 0.072</td>
<td>F:b 0.553</td>
</tr>
<tr>
<td></td>
<td>Diversity 0.332</td>
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<td></td>
<td>N 0.552</td>
<td>PCo1 0.577</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C 0.309</td>
<td>pH 0.749</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>P 0.972</td>
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</tr>
<tr>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BG</td>
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<td>0.015</td>
<td>0.004</td>
</tr>
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<td></td>
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<td>0.212</td>
<td>0.429</td>
<td>0.452</td>
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</tr>
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<td></td>
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<td>109.12</td>
<td>103.81</td>
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<td>pH &lt;0.001</td>
<td>Rich. &lt;0.001</td>
</tr>
<tr>
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<td>Evenness 0.674</td>
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</tr>
<tr>
<td></td>
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<td></td>
<td>N 0.376</td>
<td>PCo1 0.219</td>
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<td></td>
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</tr>
<tr>
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<td>P 0.437</td>
<td>Moist. 0.116</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>NAG</td>
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<tr>
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<td>0.450</td>
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<tr>
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<td>94.28</td>
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<tr>
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<td>Richness &lt;0.001</td>
<td>Bacteria 0.211</td>
<td>PCo 1 &lt;0.001</td>
<td>pH &lt;0.001</td>
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</tr>
<tr>
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<td>PCo 1 0.477</td>
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<tr>
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<td>Total resp. responses</td>
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<td>Bacteria 0.182</td>
<td>PCo 1 0.041</td>
<td>pH 0.064</td>
<td>Rich. 0.027</td>
</tr>
<tr>
<td></td>
<td>Evenness 0.925</td>
<td>Fungi 0.003</td>
<td>PCo 2 0.019</td>
<td>NO₃ 0.251</td>
<td>Fungi 0.012</td>
</tr>
<tr>
<td></td>
<td>Diversity 0.121</td>
<td>F:b ratio 0.037</td>
<td></td>
<td>N 0.010</td>
<td>F:b 0.395</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C 0.788</td>
<td>PCo1 0.660</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P 0.897</td>
<td>PCo2 0.510</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Moist. 0.185</td>
<td>N 0.270</td>
</tr>
<tr>
<td>Total resp. responses</td>
<td>Model P 0.067</td>
<td>0.004</td>
<td>0.012</td>
<td>0.059</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>Adj. R² 0.207</td>
<td>0.419</td>
<td>0.305</td>
<td>0.319</td>
<td>0.338</td>
</tr>
<tr>
<td></td>
<td>AICc 58.13</td>
<td>51.27</td>
<td>53.40</td>
<td>62.41</td>
<td>61.78</td>
</tr>
</tbody>
</table>
Table 4.4 Model fit for the final model which best explains variation of each function, for models in which the variables were dropped one by one, and for intercept-only models. Each row represents a model containing those variables for which the cells are filled. Models are ranked by declining explanatory power (Akaike weights \([w_i]\)).

<table>
<thead>
<tr>
<th>Function</th>
<th>Richness</th>
<th>Moisture</th>
<th>(R^2)</th>
<th>AIC&lt;sub&gt;c&lt;/sub&gt;</th>
<th>∆ AIC&lt;sub&gt;c&lt;/sub&gt;</th>
<th>(w_i)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-Glucosidase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Richness</td>
<td></td>
<td></td>
<td>0.578</td>
<td>96.104</td>
<td>0</td>
<td>0.758</td>
</tr>
<tr>
<td>Moisture</td>
<td></td>
<td></td>
<td>0.462</td>
<td>98.731</td>
<td>2.627</td>
<td>0.203</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.371</td>
<td>102.174</td>
<td>6.069</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>109.972</td>
<td>13.868</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>N-acetyl-glucosaminidase</strong></td>
<td></td>
<td></td>
<td>0.576</td>
<td>86.089</td>
<td>0</td>
<td>0.788</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td>0.456</td>
<td>88.848</td>
<td>2.759</td>
<td>0.198</td>
</tr>
<tr>
<td>Moisture</td>
<td></td>
<td></td>
<td>0.300</td>
<td>94.406</td>
<td>8.316</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>99.838</td>
<td>13.748</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Acid Phosphatase</strong></td>
<td></td>
<td></td>
<td>0.850</td>
<td>133.502</td>
<td>0</td>
<td>0.741</td>
</tr>
<tr>
<td>Richness</td>
<td></td>
<td></td>
<td>0.814</td>
<td>135.614</td>
<td>2.112</td>
<td>0.258</td>
</tr>
<tr>
<td>Moisture</td>
<td></td>
<td></td>
<td>0.332</td>
<td>163.784</td>
<td>30.281</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>170.251</td>
<td>36.748</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Basal respiration</strong></td>
<td></td>
<td></td>
<td>0.528</td>
<td>-61.718</td>
<td>0</td>
<td>0.886</td>
</tr>
<tr>
<td>Bacteria</td>
<td></td>
<td></td>
<td>0.353</td>
<td>-57.493</td>
<td>4.224</td>
<td>0.107</td>
</tr>
<tr>
<td>NO3</td>
<td></td>
<td></td>
<td>0.117</td>
<td>-50.628</td>
<td>11.090</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>-50.318</td>
<td>11.400</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Sum of respiration responses</strong></td>
<td></td>
<td></td>
<td>0.472</td>
<td>49.526</td>
<td>0</td>
<td>0.928</td>
</tr>
<tr>
<td>Richness</td>
<td></td>
<td></td>
<td>0.186</td>
<td>56.358</td>
<td>6.832</td>
<td>0.030</td>
</tr>
<tr>
<td>Total N</td>
<td></td>
<td></td>
<td>0.185</td>
<td>56.389</td>
<td>6.863</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>58.481</td>
<td>8.954</td>
<td>0.010</td>
</tr>
</tbody>
</table>
The bivariate correlations between significant predictors and all the soil functions investigated followed positive linear relationship (Figure 4.10). Nonlinearities between response and predictor variables were not detected by visual inspection for any of the soil functions (data not shown).
**Figure 4.10** Bivariates correlations between single variables in the best-fitting model and the activities of β-glucosidase (A, B), N-acetyl-glucosaminidase (C, D), acid phosphatase (E, F), and the rates of basal respiration (G, H) and total substrate responses (I, L).
4.3.7. Effects of forest management on soil functioning

The direct and indirect effects of forest management practices (i.e. irrigation and fertilisation) and land use (i.e. managed forest vs. natural woodland) on soil functioning were evaluated by means of structural equation modelling. Potential β-glucosidase activity was not directly influenced by land use and forest management practices (Figure 4.11A). Both bacterial richness and soil moisture were strongly positively influenced by irrigation and negatively by land use. Because richness was not significantly correlated to BG activity, indirect effects of irrigation and land use were mediated by soil moisture. Similar results were found for N-acetyl-glucosaminidase activity, where no direct effects of forest management and land use were observed, and indirect effects were driven by soil moisture, despite its correlation with NAG activity was marginally significant ($P = 0.047$). Soil pH was positively influenced by irrigation, but no significant correlation with NAG activity was observed (Figure 4.11B). Direct effects of forest management practices were observed on the potential activity of acid phosphatase (Figure 4.11C). Managed forest negatively affected AP activity, while irrigation had a positive influence on the activity this enzyme. Indirect effects were evident for land use and irrigation on bacterial richness and moisture.
Figure 4.11 Results from structural equation modelling on the predictors of β-glucosidase activity (A), N-acetyl-glucosaminidase activity (B), acid phosphatase activity (C). Arrows indicate statistically significant paths (* \( P < 0.05 \); ** \( P < 0.01 \); *** \( P < 0.001 \)). Non-significant paths are not shown. Solid and dashed arrows indicate positive and negative relationships, respectively. Arrow widths correspond to the effect size, determined by the standardised partial regression coefficients (for scale: Moisture → BG = 0.112, Management → Richness (A) = -0.982). Values in the boxes indicate \( R^2 \) associated with full model.

Direct effects of forest management practices and land use were observed for basal respiration, which was positively influenced by fertilisation and negatively by land use (Figure 4.12A). Indirect effects were mediated by bacterial abundance, which resulted positively influenced by irrigation and negatively by fertilisation and land
use. Fertilisation and land use had similar direct effects also on total substrate response (Figure 4.12B). None of the predictors of this function was significantly correlated to total respiration, therefore indirect effects of forest management practices and land use were not evident for this function.

Bacterial richness was identified as a significant predictor of BG activity and total respiration response by the model-based approach (Table 4.3), but, when analysed in the structural equation model, it was not found to be significantly correlated (Figure 4.10 and Figure 4.11). It is possible that the additional variation explained by the exogenous paths (i.e. direct effects of land use and management on responses) changed the significance of regressions in the structural equation model.

**Figure 4.12** Results from structural equation modelling on the predictors of basal respiration (A) and sum of substrate responses (B). Arrows indicate statistically significant paths (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Non-significant paths are not shown. Solid and dashed arrows indicate positive and negative relationships, respectively. Arrow widths correspond to the effect size, determined by the standardised partial regression coefficients (refer to Figure 4.11 for scale). Values in the boxes indicate $R^2$ associated with full model.
4.4. Discussion

4.4.1. Effects of forest management practices on soil microbial community structure

This study investigated the relationship between bacterial diversity and other soil biotic and abiotic factors and the performance of soil functions within a forest management experiment.

The sequencing results indicate that forest management practices influenced the composition of bacterial communities at the HFE site mainly through the separate effects of irrigation and fertilisation. In fact, bacterial communities from both the irrigated treatments (i.e. irrigation and irrigation + fertilisation) were separated from the communities from the other treatments along the major axis, which accounted for most of the variation of the system (60.63%). The communities from the fertilisation-only treatment were separated from the others along the second axis of PCoA plot, which accounted for the 18.37% of the variation. There was no clear separation of bacterial communities from NW and C treatments (Figure 4.2), suggesting that land use (i.e. plantation vs. natural woodland) had no detectable effects on bacterial community composition. This could be due to the fact that differences associated with irrigation and fertilisation treatments were stronger than differences associated to forest plantation. Soil pH, moisture and the concentration of extractable phosphate were the main drivers of bacterial community structure. As pH and soil moisture were the highest in the irrigated treatments and phosphate concentration was the highest in the plots subjected to fertilisation, this suggests that the bacterial community composition was influenced by irrigation, that increased soil pH and moisture content, and of fertilisation alone due to the higher phosphate
concentration. The increase of soil pH was directly linked to the irrigation treatment in the structural equation modelling results, and it has been reported to be correlated to microbial community structure and composition (Lauber et al., 2008, 2009). The bacterial communities in I and IF appeared to be very similar. Hence no fertilisation effect was evident in the IF treatment, suggesting that the fertilisation effects on bacterial communities of the IF plots was overcome by irrigation effects (Figure 4.2 and Figure 4.3). On the other side, bacterial communities in the F plots were different from the others and correlated with extractable phosphates, suggesting that the increased availability of phosphates due to solid fertilisation without irrigation was responsible for changes in bacterial community composition in the F plots.

Likewise, in a long-term fertilisation experiment testing the effects of N and P availability on soil microbial communities in wheat crops, Cruz et al. (2009) found that P availability selected for distinct soil microbial communities. This could be due either to a direct effect of higher soil phosphates content in the F plots or to a shift in the communities in the other treatments in favour of organisms with lower P requirements (Elser et al., 2003) caused by lower availability of P. Similar effects of irrigation and fertilisation were observed on the community structure of ammonia-oxidising archaea in a previous study carried out in the same experimental site as the current study (Hu et al., 2014). The authors observed that the community of I and IF treatments was separated from the communities of the other treatments due to pH and moisture contribution, concluding that irrigation was more important than fertilisation in impacting the microbial community (Hu et al., 2014).

Furthermore, in the present study, irrigated plots (I and IF) had the highest values of OTU richness and diversity indices, while fertilisation had the lowest, but it did not differ significantly from the control plots (i.e. C). These observations were
supported by structural equation modelling. In fact, irrigation had a strong positive effect on bacterial richness, while no significant effects of fertilisation on richness were observed. Soil pH is known to influence both microbial richness and diversity, with lower richness and diversity levels in more acidic soils (Fierer and Jackson, 2006, Lauber et al., 2009). As mentioned above, soil pH in this study was strongly influenced by irrigation. Therefore, irrigation was the most important factor influencing bacterial diversity potentially via its effects on soil pH.

The fact that irrigation effects have masked the effects of fertilisation when both treatments were applied together (IF) may be explained by the higher sensitivity to an increase of moisture content of communities developed in dry soils. In fact, there has been evidence suggesting that the effects of water availability on microbial communities are more evident in arid and semi-arid soils (Sarig and Steinberger, 1993, Austin et al., 2004).

4.4.2. Relationship between microbial communities and soil functions

Despite the fact that several controlled experiments manipulating microbial diversity found contrasting effects on soil functions (e.g. Griffiths et al., 2000, Wertz et al., 2006), observational studies of natural bacterial diversity have found a higher correlation between microbial communities and soil processes. For instance, in a study exploring relationships between soil functions and microbial populations in agroecosystems characterised by contrasting management practices and soil types, a significant correlation of cellulase activity and substrate-induced respiration with microbial composition was observed (Reeve et al., 2010). A link between microbial communities and functions was observed by another study, where the evenness of soil denitrifier communities was found to be associated with denitrification rates.
across conventional and integrated agricultural systems (Powell et al., 2015). The model-based approach used here to estimate the independent contributions of microbial community properties to soil functioning uphold previous findings (Powell et al., 2015, Reeve et al., 2010), supporting the hypothesis that soil functioning is linked to microbial communities. Most of the soil functions investigated here were, in fact, predicted by parameters representing bacterial diversity and abundance. The edaphic factors measured in this work also played a significant role in driving the soil functions, in particular soil moisture. Applying this model-based approach to these field observations provided the evidence that a positive liner relationship between microbial communities and ecosystem functioning exists, and hence changes in diversity can have direct effects on soil processes. Edaphic properties also contributed significantly to the overall functioning, supporting the importance of soil properties in influencing functioning in soil ecosystems highlighted by previous studies (Kuan et al., 2007).

In a manipulative study (Griffiths et al., 2000), the artificial reduction of microbial diversity did not affect broad-scale functions (e.g. decomposition) but it reduced rates of more specific functions (methane oxidation, nitrification, denitrification). In this field study, I observed significant positive correlations between microbial richness and processes involved in nutrient cycling, which are not exclusively carried out by narrow groups of microorganisms. Therefore, the functional redundancy hypothesised for general microbial functions (Schimel, 1995) is not supported by these results. This seems to support the current criticisms raised against manipulative controlled BEF experiments, according to which the saturation of ecosystem functioning with increasing species number is largely an artefact of homogeneous...
conditions and unrealistic assemblages of species, typical of these experiments. In fact, theory developed in plant ecology predicts that the influence of diversity increases in heterogeneous environments (Tilman et al., 1997b, Cardinale et al., 2000). Evidence from several natural, non-experimental systems supports this prediction (Tylianakis et al., 2008). Therefore, biodiversity may have its greatest impact in the functioning of naturally diverse and heterogeneous ecosystems, such as soil.

4.4.3. Effects of forest management practices on soil ecosystem functioning

In this study, I investigated the direct and indirect effects of land use and land management practices on soil potential ecosystem functioning by analysing the influence of forest plantation vs. natural woodland, and of irrigation and fertilisation on the soil functions and their predictors. The results of structural equation modelling showed that management directly affected soil respiration and the activity of acid phosphatase, while its indirect effects were mediated by a negative influence on bacterial richness. The mechanisms through which the land-use change from the natural woodland to managed forest may affect the soil microbial community and its functioning might be related to the change in tree species and the disturbance associated with plantation establishment and subsequent management practices. Shifts in tree species, in fact, may result in changes in the quality and quantity of organic matter inputs via rhizodeposition and litter (Priha and Smolander, 1997, He et al., 2006). The natural woodland of this study is composed of mixed vegetation, with patches of distinctly different plant composition, often with diverse understory vegetation. In contrast, the managed forest is a plantation of a single eucalypt species and predominately single species (i.e. Elymus repens) grass understory (Frew et al.,
Past studies have found a decline in microbial biomass associated with land-use change from native forest to plantations, as well as differences in soil respiration associated with different tree species (Waldrop et al., 2000, Chen et al., 2004). In a study examining the impact of land-use change from native forest to pine plantation in subtropical Australia, reductions in soil microbial biomass, carbon source utilisation and respiration were observed in the managed forest compared to the unmanaged control (Burton et al., 2010). Because litter and soil had lower C/N ratios in the natural forest than in the plantation, the reduction in soil respiration and carbon utilisation and changes in soil microbial community composition were suggested to be a consequence of reductions in the quality and quantity of organic matter inputs associated with the land-use change (Burton et al., 2010). Similarly, nutrient-related factors correlated with litter input and explaining a significant proportion of the variation in carbon sources utilisation were observed to be more favourable in natural forest compared to pine plantation in a similar study in Southern China (Wang et al., 2011). Therefore, the negative influence of managed forest on soil functions observed in this study might be interpreted as the effect of the change in vegetation, which caused a decrease in quantity and quality of organic matter available for decomposition by the microbial community in the managed forest. Because I did not measure C/N ratios in litter, this study does not provide empirical evidence of the effect of vegetation, and therefore further work is needed to confirm this.

According to structural equation modelling results fertilisation had a positive direct effect on basal respiration and total carbon substrate utilisation. This finding is in contrast to the majority of studies showing a reduction in respiration with N
additions (Janssens et al., 2010). However, other studies have shown that N additions can stimulate soil respiration (Cleveland and Townsend, 2006, Yoshitake et al., 2007, Hasselquist et al., 2012). These discrepancies in the observed responses may reflect differences in site characteristics, with respiration decreasing with N additions in N-rich environments (Cusack et al., 2011) and increasing in N-limited forests (He et al., 2006, Hyvönen et al., 2007, Gao et al., 2014). The influence of nutrient availability on microbial function may also depend on the fertiliser application rate, and the application rate of 150 kg N ha\(^{-1}\) year\(^{-1}\) in this experiment may not have been high enough to affect soil respiration and C sources utilisation, as it did not produce significant changes in the levels of nitrates in soils. Berch et al. (2006) reported that annual fertilisation for 10 years with 50–100 kg N ha\(^{-1}\) year\(^{-1}\) increased microbial activity and microbial diversity in a pine forest, but a higher N application (100–200 kg ha\(^{-1}\) year\(^{-1}\)) depressed microbial communities.

Irrigation had strong indirect effects on all soil functions, mediated by its positive influence on soil moisture (BG and NAG), bacterial richness (AP and total respiration), and bacterial abundance (basal respiration). Direct effects of irrigation were observed on AP activity. These results, together with the influence on bacterial community composition, highlight the significance of water availability for soil microbial communities and functioning in the dryland forest ecosystem investigated. Moisture content of control and fertilisation treatments at the time of sampling was about 3%, while in the other treatments it was about 10%. In this dry environment, the increase in water availability induced by the irrigation treatment might have greater effects on both microbial communities and functioning. In fact, water availability has been pointed out as the most important factor influencing greenhouse
gas fluxes mediated by microbes (Martins et al., 2015) and the metabolic activity of ammonia oxidisers (Hu et al., 2015) in this dryland ecosystem.

Furthermore, in dryland ecosystems, species richness of plants, mammals and birds has been found to be correlated with water availability by a linear relationship (Li et al., 2013), suggesting that the availability of water can represent a main constraint for biodiversity. This study provides evidence that this observation is valid also for soil bacterial communities in a dryland forest ecosystem, where changes in richness can have consequences for soil processes.

4.5. Conclusions and future directions

Previous biodiversity-ecosystem functioning experiments manipulating microbial diversity have shown contrasting results, and it is commonly thought that microbial communities are highly redundant (Wertz et al., 2006). The observation of natural below-ground communities and processes provided the evidence that microbes are important drivers of ecosystem functioning in forest soils. Bacterial richness and abundance, and soil functions involved in nutrient cycling were linked by significant linear relationships. This study demonstrated that variation in microbial communities under natural conditions had functional consequences for soil processes, showing that biodiversity can have important effects on ecosystem functioning in the real world. Despite this study presents statistical correlations that cannot be used to infer effects of biodiversity loss per se, our results provide empirical evidence that a positive BEF relationship occurs in these soils. Land-use change from natural woodland to managed forest had negative effects on both bacterial communities and soil functions. Within the managed forest plots, irrigation positively influenced soil functions through increasing water availability and soil pH, and bacterial richness.
Therefore, effects of land-use change and land management practices may have significant implications in forest systems for the long-term provision of ecosystem services by the soil resource.
CHAPTER 5

Microbial diversity and functional stability in natural soil communities
5.1. Introduction

The effect of a disturbance on ecosystem stability can be separated into two components: resistance and resilience. Resistance is defined as the capacity to withstand disturbance, and resilience is defined as the capacity to recover after disturbance (Pimm, 1984). Understanding stability to disturbance is an integral part of the assessment of the sustainability of an ecosystem. Soil is a non-renewable resource and it is increasingly under environmental pressure related to the intensification of human activities (Creamer et al., 2010). Microorganisms living in soil are abundant, highly diverse, and key players of many soil functions (Curtis et al., 2002, Gans et al., 2005). Therefore, in order to ensure the maintenance of soil functions, we need to fully understand the response of soil microbial communities to disturbance.

Diverse communities are believed to promote stability of ecosystem processes and biomass productivity (Tilman, 1996). Many studies focused on plant ecology have investigated the relationship between diversity and stability of ecosystem processes, concluding that increasing species richness stabilises the biomass of whole communities (Hector et al., 2010, Campbell et al., 2011). One of the possible consequences of biodiversity loss, proposed by the insurance hypothesis (Yachi and Loreau, 1999), is the reduction of ecosystem stability. This is based on the idea that the probability of finding species able to adapt to changed environmental conditions is greater in more diverse ecosystems. This hypothesis has been tested in soil by decreasing microbial diversity using chloroform fumigation: soils with the lowest diversity showed reduced resilience of decomposition to heat and copper disturbance (Griffiths et al., 2000). Nonetheless, subsequent experiments manipulating the
diversity of an arable soil by sterilisation and inoculation with diluted soil suspensions (Griffiths et al., 2001b), or of a grassland soil by both fumigation or dilution (Griffiths et al., 2004), found no effects of biodiversity on stability. This may have been caused by the way biodiversity was manipulated, which can affect the response of soil microbial communities to disturbance (De Ruiter et al., 2002). For instance, fumigation may have selected for more resistant species (Griffiths et al., 2000, 2001b) and therefore stability is thought to be related to physiological traits of individual species (Griffiths et al., 2004).

In the previous experimental demonstration of functional stability reported in chapter 2, the diversity of a soil microbial community was artificially manipulated under laboratory conditions. While the tenfold dilution approach allows the effective reduction of the diversity of a microbial community, it can impact the community structure in a way that may not be representative of what happens in soils following disturbance. The objective of this study was to determine stability to perturbation of field soils with naturally different levels of biodiversity. Soils were analysed within an existing forest management experiment and in natural woodland sites associated with different vegetation assemblages. As demonstrated in chapter 4, the soils of these sites harboured microbial communities with different diversity and composition, offering the possibility to investigate the relationship between diversity and stability of soil functions to perturbation. It is here hypothesised that a higher resistance and resilience of soil functions is associated with higher diversity of microbial communities.
5.2. Materials and Methods

5.2.1. Sites description and soil sampling

Soil samples were collected from the 16 plots of the Hawkesbury Forest Experiment (HFE) and from the eight sites in the adjacent natural woodland that were previously demonstrated to harbour different levels of bacterial diversity (chapter 4). Full field site description and sampling method are described in section 4.2.1. Soil characteristics are described in section 4.3.1.

5.2.2. Soil microcosms

The experiment was set up on the day that samples were collected. Each microcosm consisted of a sterile 250-ml Wheaton wide-mouth glass bottle to which 100 g of soil was added. Lids were left loose to allow gas exchange and minimise water loss during the incubation, but at the same time avoiding contamination from the atmosphere. Soil moisture content was adjusted and maintained for the duration of the experiment under sterile conditions at 10% (highest field moisture content at time of sampling) and the microcosms were pre-incubated for four days at room temperature to allow the soil communities to stabilise after sieving. Two parallel series of soil microcosms were created for this experiment. One set was incubated continuously at room temperature, representing the room temperature control, and the other set was subjected to a heat treatment, as described below, to provide a shock and reduce microbial biomass in order to investigate resistance and resilience of soil functionality to disturbance.
5.2.3. Disturbance treatment

Microcosms were incubated at 60 °C for 30 minutes. This temperature was chosen to be an appropriate shock for the soils under study. The purpose was to impart a stress hard enough to disturb the microbial community, but not too harsh to be unrealistic and to cause too high mortality. The soil at the HFE site can be exposed to high temperatures and the highest air temperature ever recorded in the area is 46.4 °C (Bureau of Meteorology, Australian Government). The total time of incubation equalled the sum of the time for the soil temperature to reach 60 °C (approximately 15 minutes) and a constant period of 30 minutes. This duration was previously determined in a parallel set of Wheaton bottles using a thermometer placed in the centre of the soil microcosms, and measuring the time required for the soil temperature to reach 60 °C. After the heat shock, the soil moisture content was checked and adjusted when necessary, and a recovery period of one month was established via incubation of microcosms at room temperature together with the control set.

5.2.5. Molecular analyses

Both sets of microcosms were sampled for molecular analyses two days after they were subjected to heat disturbance, and again at 30 days following disturbance. Total genomic DNA was isolated following the procedure described in section 2.2.4, with slight modification whereby 0.25 g of each soil sample were weighed into a single Power Soil® Bead tube. PCR amplification, T-RFLP analysis, and the calculation of diversity indices were carried out as described in section 2.2.5. Bacterial and fungal abundances were quantified by means of qPCR as described in section 3.2.3.
5.2.6. Functional measurements

Basal respiration, total substrate responses (i.e. sum of respiration responses to the addition of 11 carbon sources), and potential enzyme activities were measured in the room temperature control microcosms and in the heat shocked ones, one and 30 days after disturbance to assess the functional stability of the soils. The methodologies described in section 3.2.4 were followed.

5.2.7. Soil physicochemical analyses

The soil physicochemical analyses (exchangeable nitrate, extractable phosphate, total carbon and nitrogen, soil pH and soil moisture content) were carried out as described in section 3.2.5.

5.2.8. Statistical methods

All the statistical analyses were carried out using the software R 3.1.1 (R Core Development Team, 2014). The responses of the three diversity indices (i.e. richness, diversity and evenness) and of bacterial and fungal abundances were analysed separately at each time point using a two-way ANOVA, with management type, temperature treatment (either control or heat shock) and their interaction as explanatory variables. The dependent variables were tested for normality and homoscedasticity using the Shapiro-Wilk’s test and the Bartlett’s test, respectively. Where significant differences for the main effect were observed ($P < 0.05$), a Tukey’s pairwise comparison test was also performed. Bacterial and fungal abundances, based on qPCR, were log-transformed in order to meet the normality assumptions of the ANOVA. In analysing the indices of diversity and evenness, a
correction for heteroscedasticity using a heteroscedasticity consistent covariance matrix (HC3; Long and Ervin, 2000) was applied.

Basal respiration, total substrate responses, and potential enzyme activities collected in both series of soil microcosms were used to calculate resistance and resilience indices for each function, using the indices described in sections 2.2.7.1 and 2.2.7.2. These indices are bounded by 1 and -1, with 1 representing full resistance and resilience and -1 the lowest resistance to and recovery from disturbance (Orwin and Wardle 2004). Resistance and resilience indices were analysed separately for each ecosystem function by one-way ANOVA with management type as the explanatory variable. The assumptions of normality and homoscedasticity were tested as above, and Tukey’s pairwise comparison test was performed when significant differences were observed ($P < 0.05$).

The stability of soil functions was analysed by means of stepwise multiple regressions examining the explanatory power of microbial community variables and edaphic factors, following the approach described in section 3.2.6. The only exceptions to this method were that resistance and resilience indices calculated for each function were used as response variables, and the Principal Components Analysis (PCA) scores of the first two axes were used as predictors of bacterial community composition when the response variables were not correlated to any OTU. PCA was used because it was an appropriate ordination method for T-RFLP data, as confirmed by detrended correspondence analysis (Hill and Gauch Jr, 1980, Oksanen and Minchin, 1997).

The influence of forest management practices on the stability of soil functions was evaluated by means of structural equation modelling as described in section 4.2.5.
The effects of irrigation, fertilisation and management (managed forest vs. unmanaged natural woodland) on the significant predictors of resistance and resilience indices were analysed separately for each function. The same conceptual model of the relationship between stability (resistance and resilience separately), predictors, and management practices was applied to each function (Figure 4.1).

5.3. Results

5.3.1. Effects of disturbance on microbial communities

5.3.1.1. Diversity, richness and evenness

The T-RFLP analysis of the 16S rRNA gene carried out in control and disturbed microcosms two days after disturbance yielded a total of 142 forward and 94 reverse TRFs. Diversity indices (i.e. OTUs richness, Simpson’s diversity index, and Pielou’s index of evenness) measured two days after the heat disturbance was applied are reported in Figure 5.1. There was no statistically significant interaction between the effects of disturbance and management on OTU richness (Figure 5.1A) ($F_{4,38} = 1.407$, $P = 0.250$). A significant interaction between disturbance and management on the Simpson’s diversity index was found ($F_{4,38} = 4.380$, $P = 0.005$). Diversity in soils from the HFE control plot (i.e. C) was 10% lower in the disturbed microcosms compared to the room temperature treatment. A higher decrease in the Simpson’s index after disturbance was observed in the soils from the fertilised plot (i.e. F), which differed by 15% from the room temperature control. No statistically significant difference was observed in the remaining soils (Figure 5.1B). Similarly, a significant interaction between the effects of heat disturbance and management was
found on the evenness indices ($F_{4,38} = 18.396, P < 0.001$). For fertilised soils, evenness was 18% lower after disturbance than in the room temperature control (Figure 5.1C). Control soils showed a similar trend, but with a non-significant decrease of evenness of 5% after disturbance. A significant overall effect of temperature treatment was also observed ($F_{1,38} = 21.552, P < 0.001$), with evenness significantly lower in the disturbed soils.

The three diversity indices were measured again 30 days after the soil microcosms were subjected to disturbance. Results are reported in Figure 5.2. A month after heat shock disturbance, no significant interaction between the effects of heat disturbance and management on OTU richness was found ($F_{4,36} = 0.449, P = 0.771$). Similarly, there was no significant difference in richness between disturbed and control soils ($F_{1,36} = 0.507, P = 0.480$) (Figure 5.2A). A similar trend was observed for diversity (Figure 5.2B). In fact, no statistically significant interaction between the effects of disturbance and management was found ($F_{4,36} = 0.775, P = 0.548$). Also for diversity indices, there was no significant difference between disturbed and control soils ($F_{1,36} = 0.383, P = 0.539$). Thirty days after disturbance, evenness was found to remain lower in the disturbed soils, compared to the room temperature controls (Figure 5.2C), with a significant effect of heat treatment ($F_{1,36} = 5.887, P = 0.020$). No statistically significant interaction between the effects of temperature treatment and management was found on evenness ($F_{4,36} = 0.541, P = 0.706$).
Figure 5.1 Effects of heat disturbance on OTU richness (A), Simpson’s index of diversity (B), and Pielou’s index of evenness (C) calculated on T-RFLP profiles measured two days after disturbance. ‘Management’ refers to forest management treatment, ‘treatment’ refers to heat disturbance, and ‘man. x treat.’ represents the interaction between the two. NW = natural woodland; C = control; F = fertilisation; I = irrigation; IF = irrigation + fertilisation. Different letters within a treatment (i.e. control or perturbed) indicate values that are significantly different ($P < 0.05$). Error bars indicate ± one standard error (n=8 for NW, n=4 for the others). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns = not significant.
Figure 5.2 Effects of heat disturbance on OTUs richness (A), Simpson’s index of diversity (B), and Pielou’s index of evenness (C) calculated on T-RFLP profiles measured 30 days after disturbance. ‘Management’ refers to forest management treatment, ‘treatment’ refers to heat disturbance, and ‘man. x treat.’ represents the interaction between the two. NW = natural woodland; C = control; F = fertilisation; I = irrigation; IF = irrigation + fertilisation. Error bars indicate ± one standard error (n=8 for NW, n=4 for the others). * P < 0.05; ** P < 0.01; *** P < 0.001; ns = not significant.
5.3.1.2. Bacterial and fungal abundances

Bacterial and fungal communities were quantified at each time point in both disturbed and undisturbed microcosms by means of qPCR. The results are shown in Figure 5.3 and Figure 5.4. Bacterial abundances ranged from $2.49 \times 10^6$ (F) to $1.58 \times 10^7$ (NW) 16S rRNA gene copies g$^{-1}$ soil in the room temperature control soils (Figure 5.3A), and they were 23 to 56% lower immediately after (2 days) disturbance, except in IF soil, where they were slightly higher (2%). A significant negative effect of heat disturbance was found ($F_{1,38} = 7.688, P = 0.008$), but there was no significant interaction between the effects of disturbance and management on bacterial abundances ($F_{4,38} = 0.720, P = 0.583$). At the end of the recovery period (i. e. 30 days), the bacterial abundances were still 39 to 69% lower in the disturbed treatment (Figure 5.3B) across all management types, with an overall significant effect of disturbance on the 16S rRNA gene copy numbers ($F_{1,38} = 16.104, P < 0.001$). No significant interaction between disturbance and management was observed for bacterial abundance ($F_{4,38} = 0.614, P = 0.654$).
Figure 5.3 Bacterial abundances in the soils incubated continuously at room temperature (dark grey bars) and in those that were subjected to heat disturbance (light grey bars), as measured after two days (A) and 30 days (B). Bacterial abundance data of the control treatment in figure A are the same presented in figure 4.5A. ‘Management’ refers to forest management treatment, ‘treatment’ refers to heat disturbance, and ‘man. x treat.’ represents the interaction between the two. NW = natural woodland; C = control; F = fertilisation; I = irrigation; IF = irrigation + fertilisation. Error bars indicate ± one standard error (n=8 for NW, n=4 for the others). * P < 0.05; ** P < 0.01; *** P < 0.001; ns = not significant.

Fungal abundances ranged from average values of 5.22 x 10^5 (IF) to 3.08 x 10^6 18S rRNA gene copies g^-1 soil (NW) in the room temperature control, and they experienced a decrease of 13 to 70% two days after disturbance (Figure 5.4A). The heat disturbance had a significant effect (F_{1,38} = 17.991, P < 0.001), but no interaction with management was observed (F_{4,38} = 0.593, P = 0.669). A month after
the heat disturbance, the negative significant effect of disturbance on the fungal abundances remained ($F_{1,38} = 38.400$, $P = < 0.001$), with no interaction effect with management ($F_{4,38} = 0.809$, $P = 0.526$, Figure 5.4B).

**Figure 5.4** Fungal abundances in the soils incubated continuously at room temperature (dark grey bars) and in those that were subjected to heat disturbance (light grey bars), as measured after two days (A) and 30 days (B). Fungal abundance data of the control treatment in figure A are the same presented in Figure 4.5B. ‘Management’ refers to forest management treatment, ‘treatment’ refers to heat disturbance, and ‘man. x treat.’ represents the interaction between the two. NW = natural woodland; C = control; F = fertilisation; I = irrigation; IF = irrigation + fertilisation. Error bars indicates ± one standard error (n=8 for NW, n=4 for the others). * P < 0.05; ** P < 0.01; *** P < 0.001; ns = not significant.
5.3.2. Stability of soil functions in relation to disturbance

5.3.2.1. Potential enzyme activity

5.3.2.1.1. Resistance

The potential enzyme activity data collected one day after shock (see Appendix E) were used to calculate resistance indices. The values of the index calculated on the activities of the three enzymes were all high, ranging from average values of $0.69 \pm 0.08$ to $0.83 \pm 0.02$ for BG (Figure 5.5A), from $0.67 \pm 0.05$ to $0.84 \pm 0.05$ for NAG (Figure 5.5B), and from $0.72 \pm 0.04$ to $0.83 \pm 0.08$ for AP (Figure 5.5C). This indicates that the heat treatment did not produce a large change in these functions, i.e. the absolute value of potential enzyme activity in the disturbed microcosms was lower than the potential enzyme activity in the control (Figure 5.5). The effect of management regime was not observed to be significant on the resistance of the activities of $\beta$-glucosidase (BG, $F_{4,19} = 1.128, P = 0.372$), N-acetyl-glucosaminidase (NAG, $F_{4,19} = 2.422, P = 0.083$), and acid phosphatase (AP, $F_{4,19} = 0.454, P = 0.767$).
Figure 5.5 Resistance indices calculated on potential enzyme activity of β-glucosidase (A), N-acetyl-glucosaminidase (B), and acid phosphatase (C), collected one day after shock in disturbed and undisturbed soils. NW = natural woodland; C = control; F = fertilisation; I = irrigation; IF = irrigation + fertilisation. Error bars indicate ± one standard error (n=8 for NW, n=4 for the others).
5.3.2.1.2. Resilience

Resilience indices were calculated on potential enzyme activity data 30 days (RL_{30}) after the heat shock was applied (see Appendix E). The results are shown in Figure 5.6. Despite the high resistance of these functions, the values of the RL_{30} were much lower, ranging from average values of -0.16 ± 0.37 (F) to 0.18 ± 0.23 (IF) for BG (Figure 5.6A), from -0.50 ± 0.20 (F) to 0.41 ± 0.18 (IF) for NAG (Figure 5.6B), and from -0.25 ± 0.41 (F) to 0.32 ± 0.24 (C) for AP (Figure 5.6C). This indicates that, even if the disturbance did not produce a large change in the potential enzyme activity, the soil communities’ functional potential did not completely return to pre-disturbance levels a month after disturbance. The values of the RL_{30} calculated on BG activity were not statistically different (F_{4,19} = 0.414, P = 0.795), while significant differences were found in the indices calculated on NAG (F_{4,19} = 2.971, P = 0.046). In fact, C and IF were found to have higher resilience than F in the potential activity of NAG. No significant differences were observed in the values of RL_{30} calculated on AP activity (F_{4,19} = 0.765, P = 0.560; Figure 5.6C). Despite differences in BG and NAG indices were not significant, it is possible to observe that the resilience of the activity of the three enzymes shower similar patterns, with the lowest values of the index in the F treatment.
Figure 5.6 Resilience indices calculated on potential enzyme activity of β-glucosidase (A), N-acetyl-glucosaminidase (B), and acid phosphatase (C), measured one month after shock in disturbed and undisturbed soils. NW = natural woodland; C = control; F = fertilisation; I = irrigation; IF = irrigation + fertilisation. Bars with different letters differ significantly \((P < 0.05)\). Error bars indicate ± one standard error \((n=8\) for NW, \(n=4\) for the others).
5.3.2.2. Respiration

5.3.2.2.1. Resistance

The resistance of community respiration was evaluated by means of calculating resistance indices on basal respiration rates and total respiration responses measured in the room temperature control and disturbed microcosms two days after shock (see Appendix E). The results are shown in Figure 5.7. As observed for the potential enzyme activities of these soils, also the basal respiration was resistant to the heat disturbance, with average values of the index ranging from 0.29 ± 0.02 to 0.64 ± 0.05 (Figure 5.7A). Marginally significant effects of management regime were observed on the resistance index of basal respiration ($F_{4,19} = 2.878$, $P = 0.050$), with I showing higher resistance than IF treatment. The resistance index calculated on total respiration responses were in general lower than those of basal respiration, with average values ranging from 0.10 ± 0.04 to 0.48 ± 0.18 (Figure 5.7B). Even though the resistance index for total respiration was, on average four times higher in the C and F treatments than in the I and IF treatments, no significant differences were found ($F_{4,19} = 1.862$, $P = 0.158$).
Figure 5.7 Resistance indices calculated on basal respiration rates (A) and sum of substrate response rates (B) measured one day after shock in disturbed and undisturbed soils. NW = natural woodland; C = control; F = fertilisation; I = irrigation; IF = irrigation + fertilisation. Bars with different letters have a marginally significant difference ($P = 0.05$). Error bars indicate ± one standard error (n=8 for NW, n=4 for the others).

5.3.2.2.2. Resilience

The resilience of community respiration to disturbance was assessed by calculating resilience indices on basal respiration rates and total substrate responses 30 days after disturbance. The results of $RL_{30}$ for basal respiration and total substrate responses are presented in Figure 5.8. Significant differences in the resilience of soil communities were observed for both basal respiration ($F_{4,19} = 4.456$, $P = 0.010$, Figure 5.8A) and total respiration responses ($F_{4,19} = 5.048$, $P = 0.006$, Figure 5.8B).
The resilience of basal respiration was significantly higher in IF (0.86 ± 0.007) compared to I (-0.34 ± 0.11), while resilience indices of total respiration responses had significantly lower values in C (-0.49 ± 0.17) compared to IF, NW and I (ranging from 0.49 ± 0.14 to 0.41 ± 0.11).

**Figure 5.8** Resilience indices calculated on basal respiration rates (A) and sum of substrate respiration responses rates (B) measured a month after shock in disturbed and undisturbed soils. NW = natural woodland; C = control; F = fertilisation; I = irrigation; IF = irrigation + fertilisation. Bars with different letters differ significantly ($P < 0.05$). Error bars indicate ± one standard error (n=8 for NW, n=4 for the others).
5.3.3. Relationship between microbial communities and functional stability

The stability of potential enzyme activities and community respiration to disturbance was investigated by estimating the independent contribution of biotic and abiotic factors to resistance and resilience indices using a model-based approach. No individual OTU was found to have its relative abundance correlated with the resistance nor the resilience indices calculated for any of the functions investigated. Therefore, the contribution of community composition to resilience was tested by including PCA scores of the first two axes in third group of variables.

5.3.3.1. Resistance

Results of the significant predictors of resistance identified via the step-wise procedure are presented in Table 5.1. The resistance of all functions analysed, except for the activity of β-glucosidase and for basal respiration, was predicted by biotic components. Within these variables, initial fungal and bacterial abundances were not found to be significant predictors of the resistance indices calculated on any of the functions. The initial independent screening of the four classes of variables also excluded the edaphic properties from the final model.

The explanatory power of diversity components was lower than the explanatory power of composition variables for the resistance of BG, NAG, and total respiration responses (e. g. Adjusted $R^2 = 0.041$ vs 0.129; BG, Table 5.1). The opposite was observed for AP, where the class of diversity components showed a higher explanatory power than community composition (Adjusted $R^2 = 0.214$ vs 0.126; Table 5.1). Edaphic factors showed a higher explanatory power only for the resistance of basal respiration (Adjusted $R^2 = 0.107$), where the other classes of variables were not observed to explain the variation of this function.
The resistance of the activity of N-acetyl-glucosaminidase was best explained by bacterial community composition variables ($P = 0.037$, Table 5.1). The second of the PCA axes (i.e. PC2) was found to be a marginally significant predictor of the resistance of NAG ($P = 0.057$). Despite this, the importance of PC2 was low ($\Delta$AICc $= 1.63$; Table 5.2). The resistance of acid phosphatase activity was best explained by the initial community evenness ($P = 0.022$, Table 5.1), which had a higher importance for the model prediction ($\Delta$AICc $= 3.38$; Table 5.2).

The resistance of total substrate response was significantly predicted by the fungi to bacteria ratio ($P < 0.001$), which had a high importance ($\Delta$AICc $= 16.80$; Table 5.2). Also PC2 was initially found to be significantly correlated to the resistance of total substrate response, but when combined in the final model, it was no longer significant. This may have been due to indirect effects of this variable on the resistance of total respiration responses via other variables in the model or to a correlation to the resistance due to an independent response to variation in another variable.

None of the variables measured in this experiment explained the resistance of BG and basal respiration (Table 5.1). The resistance of NAG, AP and total respiration response resulted correlated to their significant predictors with positive linear relationships (Figure 5.9).
Table 5.1 Results of the multiple linear regressions constructed with each class of variables, for the resistance of each ecosystem function investigated.

<table>
<thead>
<tr>
<th>Function</th>
<th>1) Diversity components</th>
<th>2) Microbial abundances</th>
<th>3) Community composition</th>
<th>4) Edaphic factors</th>
<th>Final model</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>Var.</td>
<td>P value</td>
<td>Var.</td>
<td>P value</td>
<td>Var.</td>
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Table 5.2 Model fit for the final model which best explains variation of the resistance of each ecosystem function, for models including the significant variables, and for intercept-only models. Each row represents a model containing those variables for which the cells are filled. Models are ranked by declining explanatory power ($R^2$ and Akaike weights [wi]).

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<tr>
<th>N-acetylglucosaminidase</th>
<th>$R^2$</th>
<th>AICc</th>
<th>Δ AICc</th>
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<table>
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<tbody>
<tr>
<td>F:b ratio</td>
<td>0.550</td>
<td>-79.331</td>
<td>0</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>-62.530</td>
<td>16.800</td>
<td>0.0002</td>
</tr>
</tbody>
</table>
Figure 5.9 Bivariate correlations between single variables in the full model and resistance (RS) index of N-acetyl-glucosaminidase (A), acid phosphatase (B) activity, and sum of respiration responses (C).

5.3.3.2. Resilience

Results of the significant predictors of resilience identified via the step-wise procedure are presented in Table 5.3. Similarly to what was observed for resistance,
initial fungal and bacterial abundances were not significant predictors of the resilience indices calculated on any of the functions. Diversity components showed the highest explanatory power of resilience indices calculated on β-glucosidase and acid phosphatase activities (Adjusted $R^2 = 0.114$ and 0.104 respectively, Table 5.3), and on basal respiration (Adjusted $R^2 = 0.163$, Table 5.3). Abiotic variables had the lowest power in explaining the resilience of all functions except for N-acetyl-glucosaminidase (Table 5.3). When analysing the resilience of NAG, in fact, edaphic variables were found to have the highest explanatory power (Adjusted $R^2 = 0.266$, Table 5.3). Community composition variables were significant predictors of the resilience of total substrate response only.

When analysing the resilience of NAG and AP, the initial step-wise procedure identified total soil carbon (C) as a significant predictor of the resilience of the activity of these enzymes, but C was no longer significant when combined with other factors in the final model (Table 5.3). This suggests that total carbon may have had indirect effects on the resilience of NAG and AP via other edaphic variables included in the model or may have covaried with these other variables that were important predictors of the activities of these enzymes.

The resilience of the activity of BG was explained only by initial community evenness ($P = 0.041$, Table 5.3). For NAG activity, the abiotic variables had the highest explanatory power ($P = 0.043$, Adjusted $R^2 = 0.266$; Table 5.3). Soil pH and initial community evenness were found to be significant predictors of this response ($P = 0.018$ and 0.010, respectively). Of these predictors, pH was found to be the most important ($\Delta$AICc = 4.134, Table 5.4). The resilience of AP activity was best explained by the initial community evenness and the total soil C (Table 5.3),
whereby only evenness was observed to be significantly correlated with the response in the final model ($P = 0.027$ and $0.668$ for evenness and total carbon respectively).

The model including diversity components was the best predictor of resilience of basal respiration ($P = 0.089$, Adjusted $R^2 = 0.163$, Table 5.3). Initial OTU richness was found to be a significant predictor of the resilience of basal respiration ($P = 0.048$). PCA axes were found to be the only significant predictors of the resilience of the total substrate response ($P = 0.003$; Table 5.3), with PC2 being the most important predictor ($\Delta AICc = 5.476$, Table 5.4).

In the step-wise procedure, fungal:bacterial ratio was found to be significantly correlated to the resilience of total respiration response ($P = 0.041$). When combined together with the other significant variables in the final model, fungal:bacterial ratio was no longer significant ($P = 0.139$).
Table 5.3 Results of the multiple linear regressions constructed with each class of variables, for the resilience of each ecosystem function investigated.

<table>
<thead>
<tr>
<th>Function</th>
<th>1) Diversity components</th>
<th>2) Microbial abundances</th>
<th>3) Community composition</th>
<th>4) Edaphic factors</th>
<th>Final model</th>
</tr>
</thead>
<tbody>
<tr>
<td>BG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Richness</td>
<td>0.664</td>
<td>Bacteria 0.524</td>
<td>PC1 0.152</td>
<td>pH 0.221</td>
<td>Even. 0.041</td>
</tr>
<tr>
<td>Evenness</td>
<td>0.042</td>
<td>Fungi 0.666</td>
<td>PC2 0.421</td>
<td>NO₃ 0.985</td>
<td></td>
</tr>
<tr>
<td>Diversity</td>
<td>0.313</td>
<td>F:b ratio 0.444</td>
<td></td>
<td>N 0.562</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C 0.226</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P 0.615</td>
<td></td>
</tr>
<tr>
<td>BG Model P</td>
<td>0.148</td>
<td>0.750</td>
<td>0.259</td>
<td>0.592</td>
<td>0.041</td>
</tr>
<tr>
<td>BG Adj. R²</td>
<td>0.114</td>
<td>-0.083</td>
<td>0.036</td>
<td>-0.055</td>
<td>0.138</td>
</tr>
<tr>
<td>BG AICc</td>
<td>-29.6</td>
<td>-24.79</td>
<td>-29.3</td>
<td>-21.12</td>
<td>-33.5</td>
</tr>
<tr>
<td>NAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Richness</td>
<td>0.918</td>
<td>Bacteria 0.949</td>
<td>PC1 0.087</td>
<td>pH 0.010</td>
<td>Even. 0.010</td>
</tr>
<tr>
<td>Evenness</td>
<td>0.010</td>
<td>Fungi 0.679</td>
<td>PC2 0.280</td>
<td>NO₃ 0.912</td>
<td>pH 0.018</td>
</tr>
<tr>
<td>Diversity</td>
<td>0.310</td>
<td>F:b ratio 0.740</td>
<td></td>
<td>N 0.888</td>
<td>C 0.734</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C 0.038</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P 0.687</td>
<td></td>
</tr>
<tr>
<td>NAG Model P</td>
<td>0.052</td>
<td>0.960</td>
<td>0.133</td>
<td>0.056</td>
<td>0.010</td>
</tr>
<tr>
<td>NAG Adj. R²</td>
<td>0.211</td>
<td>-1.133</td>
<td>0.095</td>
<td>0.266</td>
<td>0.335</td>
</tr>
<tr>
<td>NAG AICc</td>
<td>-32.5</td>
<td>-23.8</td>
<td>-31.0</td>
<td>-29.9</td>
<td>-36.6</td>
</tr>
<tr>
<td>AP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Richness</td>
<td>0.287</td>
<td>Bacteria 0.874</td>
<td>PC1 0.199</td>
<td>pH 0.317</td>
<td>Even. 0.027</td>
</tr>
<tr>
<td>Evenness</td>
<td>0.053</td>
<td>Fungi 0.390</td>
<td>PC2 0.626</td>
<td>NO₃ 0.861</td>
<td>C_ 0.688</td>
</tr>
<tr>
<td>Diversity</td>
<td>0.593</td>
<td>F:b ratio 0.439</td>
<td></td>
<td>N 0.951</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C 0.036</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P 0.940</td>
<td></td>
</tr>
<tr>
<td>AP Model P</td>
<td>0.162</td>
<td>0.704</td>
<td>0.384</td>
<td>0.330</td>
<td>0.077</td>
</tr>
<tr>
<td>AP Adj. R²</td>
<td>0.104</td>
<td>-0.073</td>
<td>&lt; 0.001</td>
<td>0.050</td>
<td>0.141</td>
</tr>
<tr>
<td>AP AICc</td>
<td>-25.0</td>
<td>-20.7</td>
<td>-24.1</td>
<td>-19.34</td>
<td>-27.8</td>
</tr>
<tr>
<td>Basal respiration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Richness</td>
<td>0.045</td>
<td>Bacteria 0.442</td>
<td>PC1 0.287</td>
<td>pH 0.876</td>
<td>Rich. 0.048</td>
</tr>
<tr>
<td>Evenness</td>
<td>0.148</td>
<td>Fungi 0.452</td>
<td>PC2 0.401</td>
<td>NO₃ 0.165</td>
<td></td>
</tr>
<tr>
<td>Diversity</td>
<td>0.422</td>
<td>F:b ratio 0.308</td>
<td></td>
<td>N 0.469</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C 0.581</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P 0.109</td>
<td></td>
</tr>
<tr>
<td>Basal respiration Model P</td>
<td>0.089</td>
<td>0.527</td>
<td>0.397</td>
<td>0.366</td>
<td>0.048</td>
</tr>
<tr>
<td>Basal respiration Adj. R²</td>
<td>0.163</td>
<td>-0.031</td>
<td>-0.003</td>
<td>0.033</td>
<td>0.127</td>
</tr>
<tr>
<td>Basal respiration AICc</td>
<td>-29.0</td>
<td>-24.0</td>
<td>-26.4</td>
<td>-21.2</td>
<td>-31.2</td>
</tr>
<tr>
<td>Total resp. responses</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Richness</td>
<td>0.264</td>
<td>Bacteria 0.256</td>
<td>PC1 0.020</td>
<td>pH 0.206</td>
<td>PC1 0.017</td>
</tr>
<tr>
<td>Evenness</td>
<td>0.867</td>
<td>Fungi 0.331</td>
<td>PC2 0.008</td>
<td>NO₃ 0.535</td>
<td>PC2 0.007</td>
</tr>
<tr>
<td>Diversity</td>
<td>0.260</td>
<td>F:b ratio 0.041</td>
<td></td>
<td>N 0.200</td>
<td>F:b ratio 0.139</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C 0.826</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P 0.558</td>
<td></td>
</tr>
<tr>
<td>Total resp. responses Model P</td>
<td>0.460</td>
<td>0.100</td>
<td>0.003</td>
<td>0.527</td>
<td>0.004</td>
</tr>
<tr>
<td>Total resp. responses Adj. R²</td>
<td>-0.013</td>
<td>0.151</td>
<td>0.355</td>
<td>-0.031</td>
<td>0.395</td>
</tr>
<tr>
<td>Total resp. responses AICc</td>
<td>-20.0</td>
<td>-24.3</td>
<td>-32.7</td>
<td>-15.3</td>
<td>-32.4</td>
</tr>
</tbody>
</table>
Table 5.4 Model fit for the final model which best explains variation of the resilience of each ecosystem function, for models in which the variables were dropped one by one, and for intercept-only models. Each row represents a model containing those variables for which the cells are filled. Models are ranked by declining explanatory power ($R^2$ and Akaike weights [$w_i$]).

<table>
<thead>
<tr>
<th>β-Glucosidase</th>
<th>$R^2$</th>
<th>AIC$_c$</th>
<th>Δ AIC$_c$</th>
<th>$w_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evenness</td>
<td>0.175</td>
<td>-33.554</td>
<td>0</td>
<td>0.755</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>-31.303</td>
<td>2.250</td>
<td>0.244</td>
</tr>
<tr>
<td>N-acetylglucosaminidase</td>
<td>$R^2$</td>
<td>AIC$_c$</td>
<td>Δ AIC$_c$</td>
<td>$w_i$</td>
</tr>
<tr>
<td>Evenness</td>
<td>0.418</td>
<td>-39.429</td>
<td>0</td>
<td>0.741</td>
</tr>
<tr>
<td>pH</td>
<td>0.259</td>
<td>-36.253</td>
<td>3.176</td>
<td>0.151</td>
</tr>
<tr>
<td></td>
<td>0.229</td>
<td>-35.294</td>
<td>4.134</td>
<td>0.093</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>-31.419</td>
<td>8.009</td>
<td>0.013</td>
</tr>
<tr>
<td>Acid Phosphatase</td>
<td>$R^2$</td>
<td>AIC$_c$</td>
<td>Δ AIC$_c$</td>
<td>$w_i$</td>
</tr>
<tr>
<td>Evenness</td>
<td>0.209</td>
<td>-30.256</td>
<td>0</td>
<td>0.836</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>-26.993</td>
<td>3.263</td>
<td>0.147</td>
</tr>
<tr>
<td>Basal respiration</td>
<td>$R^2$</td>
<td>AIC$_c$</td>
<td>Δ AIC$_c$</td>
<td>$w_i$</td>
</tr>
<tr>
<td>Richness</td>
<td>0.165</td>
<td>-31.291</td>
<td>0</td>
<td>0.727</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>-29.330</td>
<td>1.961</td>
<td>0.272</td>
</tr>
<tr>
<td>Sum of respiration responses</td>
<td>$R^2$</td>
<td>AIC$_c$</td>
<td>Δ AIC$_c$</td>
<td>$w_i$</td>
</tr>
<tr>
<td>PC1</td>
<td>0.411</td>
<td>-32.702</td>
<td>0</td>
<td>0.801</td>
</tr>
<tr>
<td></td>
<td>0.236</td>
<td>-29.059</td>
<td>3.643</td>
<td>0.129</td>
</tr>
<tr>
<td></td>
<td>0.175</td>
<td>-27.226</td>
<td>5.476</td>
<td>0.051</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>-24.980</td>
<td>7.722</td>
<td>0.016</td>
</tr>
</tbody>
</table>

The correlations between the significant predictors and resilience followed negative linear relationships for most of the functions (Figure 5.10). The exceptions to this were represented by the relationship between pH and the resilience of NAG activity (Figure 5.10D), and between PC1 scores and total respiration response (Figure 5.10F), both following a positive trend. Nonlinearities between response and predictor variables were not detected by visual inspection for any of the soil functions (data not shown).
Figure 5.10 Bivariate correlations between single variables in the full model and resilience index (RL₃₀) of β-glucosidase (A), acid phosphatase (B), N-acetyl-glucosaminidase (C, D) activity, and basal respiration (E) and sum of respiration responses (F,G).
5.3.4. Effects of forest management on the stability of soil functions

Structural equation modelling was used to account for the indirect effects of land use and forest management practices on the resistance and resilience of the soil functions via the variables measured here. The results are presented in Figure 5.11 and Figure 5.12.

The resistance of NAG activity was not directly influenced by land use and forest management practices. PC2 scores were positively influenced by land use, and negatively by irrigation and fertilisation treatments (Figure 5.11A). PC2 scores were not found significantly correlated to the resistance of NAG activity when assessed in the structural equation model ($P = 0.204$), despite it was identified as predictor of this response by the model-based approach. This is probably due to the marginal non significance of PC2 ($P = 0.057$, Table 5.1) in the final model of the multiple regressions explaining the resistance of NAG. The resistance of AP activity was not directly and indirectly influenced by land use and forest management practices. There were, in fact, no effects on evenness, which therefore was the only driver of the response in this model (Figure 5.11B). The resistance of total substrate response was also not directly influenced by land use and forest management, but indirect effects of irrigation and land use were observed on fungal:bacterial ratio (Figure 5.11C).
Figure 5.11 Results from structural equation modelling on the predictors of resistance indices of N-acetyl-glucosaminidase activity (A), acid phosphatase activity (B), and sum of respiration responses (C). Arrows indicate statistically significant paths (* \( P < 0.05 \); ** \( P < 0.01 \); *** \( P < 0.001 \)). Non-significant paths are not shown. Solid and dashed arrows indicate positive and negative relationships, respectively. Arrow widths correspond to the effect size, determined by the standardised partial regression coefficients (refer to Figure 4.11 for scale). Values in the boxes indicate \( R^2 \) associated with full model.

The resilience of BG activity was not directly nor indirectly influenced by land use and forest management practices (Figure 5.12A). No effects were observed on bacterial community evenness, which was hence driving the resilience of BG in the model. No direct effects of land use and forest management were observed on the resilience of NAG activity. Irrigation and fertilisation had significant opposite effects on soil pH (Figure 5.12B): irrigation positively influenced pH (\( P < 0.001 \)), while fertilisation had a weaker negative effect on pH (\( P = 0.048 \)). The resilience of NAG activity appeared to be driven by community evenness which was strongly correlated
to this response with a negative relationship. Similarly to what observed for the resistance of AP, its resilience was not directly and indirectly influenced by land use and forest management practices. No effects where observed on the predictor variable (*i.e.* evenness), hence bacterial community evenness was the only driver of the response in this model. In contrast, the influence of evenness was, in this case, negative (Figure 5.12C).

Land use and forest management effects were observed on the resilience of respiration measurements. The resilience of basal respiration was positively influenced by fertilisation treatment, while no indirect effects mediated by OTU richness were observed (Figure 5.12D). Direct effects of land use and management practices were found also on the resilience of total substrate response, which was negatively influenced by land use and positively by irrigation (Figure 5.12E). None of the predictors of this function was significantly correlated to total respiration, therefore indirect effects of forest management practices and land use were not evident for this function.

Overall, a fairly large proportion of the variance of resistance indices was explained by the model, ranging from 24 to 55% (Figure 5.11), while the proportion of the variance of resilience indices was lower (18 to 43%, Figure 5.12).

Some of the variables that were identified as significant predictors of resilience by the multiple regressions of the model-based approach were not found to be significantly correlated when assessed in the structural equation model. Within these, soil pH was a significant predictor of the resilience of NAG activity in the final model (*P* = 0.018, Table 5.3), but was not found to be significantly correlated to this response in the structural equation model (*P* = 0.087). This could be due to additional variation explained by the exogenous paths (*i.e.* direct effects of land use...
and management on responses) that may have changed the significance of regressions. Similarly, OTU richness was marginally significantly correlated to the resilience of basal respiration \( (P = 0.048, \text{Table 5.3}) \) in the final model and this correlation was not found significant in the structural equation model \( (P = 0.071) \). Similarly, PC1 and PC2 scores resulted as significant predictors of the resilience of total respiration when analysed by multiple regressions \( (P = 0.017 \text{ and } 0.007 \text{ respectively, Table 5.3}) \), but their relationships were not significant as for the structural equation modelling \( (P = 0.118 \text{ and } 0.448 \text{ respectively}) \).
Figure 5.12 Results from structural equation modelling on the predictors of resilience indices of β-glucosidase activity (A), N-acetyl-glucosaminidase activity (B), acid phosphatase activity (C), basal respiration (D), and sum of respiration responses (E). Arrows indicate statistically significant paths (* P < 0.05; ** P < 0.01; *** P < 0.001). Non-significant paths are not shown. Solid and dashed arrows indicate positive and negative relationships, respectively. Arrow widths correspond to the effect size, determined by the standardised partial regression coefficients (refer to Figure 4.11 for scale). Values in the boxes indicate R² associated with full model.
5.4. Discussion

5.4.1. Effects of disturbance on bacterial diversity and functioning

This study examined the resistance and resilience of microbial communities associated with forest soils under different management practices and land uses to a short temperature disturbance. Heating did not have an immediate effect on OTU richness, whose values were not statistically different from those of the unheated control, but it negatively affected the diversity of soils subjected to fertilisation (F) and soils from the control plots (C). Since richness did not change and a decrease of evenness was observed in these soils, we can conclude that a change in relative abundances occurred in the bacterial communities of F and C. Nonetheless, although microcosms were sampled for molecular analyses two days after the disturbance to minimise the detection of DNA of dead cells by the T-RFLP, it remains possible that these results included a part of the bacterial population that did not survive the shock. A month after the disturbance was applied, both richness and diversity recovered to values statistically similar to the undisturbed controls in all soils, while the increase in dominance caused by the disturbance did not return to pre-disturbance levels. In contrast with these findings, in a microcosms study that manipulated the microbial diversity by the dilution approach a transient heat disturbance impacted the bacterial community structure disregarding of the diversity level (Tardy et al., 2014). The different results of these two studies could be ascribed to the different length of the heat disturbance. In fact, in Tardy et al. (2014) the microcosms were treated for 24 hours at 50 °C, while in the present study we shocked the soils for 30 minutes at a higher temperature (60 °C). It is therefore possible that the shock applied in this study was not harsh enough to impact bacterial community structure. Moreover, the
methods used for assessing the community response to heat treatment were also different: Tardy et al. (2014) used 454 sequencing, a technique with higher resolution than the T-RFLP analysis used in the present study.

The potential enzyme activity showed a delayed response to the heat shock. In fact, the activity of the three enzymes showed high immediate resistance (i.e. small change) to the disturbance, but lower resilience (i.e. small recovery) a month later. Hence, even if the heat shock did not produce a significant change in the potential enzyme activity of the soils, this functionality did not recover completely to control levels during a month. The heat treatment also produced a reduction in bacterial and fungal biomass that did not show any recovery to the control levels during the incubation time. Because the resilience depends on the survival and proliferation of microbial populations capable of producing new enzymes, the slower recovery of enzyme activity measured by the resilience index can be due to the decrease in microbial abundances due to heat treatment. Alternatively, the small change in activity after the heat shock represented by the high values of resistance index might be interpreted by the increased activity of the surviving population induced by a higher availability of nutrients released from dead biomass, and by the reduction of competition for resources. This confirms previous findings in forest soils treated at 60 °C and 70 °C, where the biomass that persisted after the shock had a higher enzymatic activity than the original biomass (Chaer et al., 2009).

The ability to degrade different carbon sources showed higher sensitivity to heat disturbance than basal respiration. The total respiration response to carbon amendments had in fact lower resistance and resilience. This may be explained with a loss of specialised degraders caused by the disturbance that may have impaired the
overall utilisation of substrates by the communities, resulting in lower resistance and resilience indices. None of the two measurements of community respiration recovered to the control levels, since the resilience indices were still low 30 days after shock for both basal respiration and total substrate response. Basal respiration recovered to values similar to the control only for the IF soil, as shown by the higher RL_{30} index \( (i.e. \ 0.86 \pm 0.007) \). Similarly, Tardy \textit{et al.} (2014) observed in microcosms study that soil basal respiration did not recover to the levels of the control up to 60 days of incubation after a heat disturbance at 50 °C for 24 hours. The lower respiration was ascribed to nutrient depletion that occurred during the heating event due to increased microbial activity (Tardy \textit{et al.}, 2014). The low resilience indices observed in the present study could be explained by the same mechanisms. In addition, as discussed for the recovery of potential enzymatic activity, the decrease of microbial abundances compared with control at the end of the incubation may also have contributed to the low resilience of basal respiration.

In a study investigating stability of substrate-induced respiration in several grassland, arable and forest soils (Gregory \textit{et al.}, 2009), it was observed that a transient heat stress (40 °C) affected over a month time some soils and not others, disregarding of the vegetation covering. So it is therefore possible that other factors are responsible for the lower resilience to heat disturbance of respiration, such as physico-chemical properties of soils.

\textbf{5.4.2. Relationship between bacterial communities and stability}

The relationship between microbial communities and stability to disturbance has been mostly investigated by manipulative experiments. Studies that manipulated soil microbial diversity either by chloroform fumigation (Griffiths \textit{et al.}, 2000) or serial
dilutions (Griffiths et al., 2001b) did not find any consistent effect of biodiversity on functional stability measured as decomposition of added plant material. The same lack of relationship between diversity and resistance and resilience to heat disturbance was observed also for more specific functions, such as denitrification and nitrite oxidation (Wertz et al., 2007). Griffiths et al. (2008), concluded from an experimental manipulation of the microbial communities of different soil types that soil biological stability to perturbation depends on the physico-chemical structure of the soil. In natural soils, a much larger number of environmental factors, both biotic and abiotic, can influence the stability of soil ecosystem functions and it is therefore necessary to include them in predictive models. In the present study, field soils were perturbed under laboratory conditions and the functional stability was analysed by means of a model-based approach that allowed determination of the contributions of single classes of predictors of resistance and resilience to disturbance. Bacterial diversity components (i.e. richness, diversity and evenness) and community composition had the highest explanatory power when analysing the resistance and resilience of all soil functions, except for the resistance of basal respiration and the resilience of NAG activity, which were better explained by edaphic factors (Table 5.1 and 5.3). These results are in contrast with the findings of studies manipulating diversity reported above, but they are comparable to experimental perturbations of natural soils. For instance, in forest soils, resistance of substrate-induced respiration to heat, tested in a similar laboratory experiment, was not found to be significantly related to soil properties, but to bacterial community structure (Banning and Murphy, 2008). No correlation was observed in a wide range of soil types and land management between resistance and resilience of plant residues decomposition to a transient heat stress and soil properties such as pH, texture, and carbon and nitrogen
contents (Kuan et al., 2007). Our results demonstrate that microbial communities are important drivers of the stability to disturbance of some of the soil functions investigated, independently from the abiotic properties considered. Therefore, this indicates that changes in the diversity and composition of microbial communities may have consequences for the ability of soil to resist and recover from environmental perturbations.

The initial evenness of the bacterial community was the only significant predictor within the diversity components class of the resistance and resilience of potential enzymatic activity. Community evenness has been previously demonstrated to be significantly correlated to functionality under disturbance in a microcosms study manipulating the evenness of denitrifiers (Wittebolle et al., 2009), and under field conditions along an existing gradient in denitrifier evenness (Powell et al., 2015). In our study, a positive effect of evenness was observed on resistance of enzyme activity, while evenness and resilience were negatively correlated. When in a community species are functionally redundant and the initial evenness is high (i. e. the individuals are equally distributed among species), the probability that a species tolerant to a perturbation is present is higher than when evenness is low. When the community is highly dominated by one or a few species, resistance to the perturbation will only occur if the dominant species are tolerant to the perturbation (Wittebolle et al., 2009), explaining the positive correlation between evenness and resistance. On the other hand, if the community evenness is high and more tolerant species survive the disturbance, interspecific competition may disfavour the recovery of functionality, resulting in a decrease in resilience, explaining the negative correlation between evenness and resilience.
Within the diversity components class, initial bacterial richness was a significant predictor of the resilience of basal respiration. Contrarily to what hypothesised, the relationship between richness and resilience was negative, indicating that the recovery after disturbance is higher in communities with a lower number of bacterial species. In addition to positive interactions, biodiversity may increase competition between organisms within communities (Polley et al., 2003, Jousset et al., 2011). It has been observed that competition for resources can affect ecosystem functioning if inefficient species become dominant (negative complementary effect, Jiang et al., 2008), and by inhibiting efficient competitors (Massey et al., 2004). When a disturbance occurs in a community dominated by antagonistic interactions, the functioning can be negatively affected, resulting in a slower recovery of functionality. Therefore, the negative relationship between richness and resilience observed in this study may be interpreted as the result of antagonistic interactions in the microbial communities. These findings suggest that bacterial diversity and community composition play an important role in the ability of the community to resist and recover from a disturbance event. In soil ecosystems, complex and inverse relationships may exist between resistance and resilience (Orwin et al., 2006). Our results showed that variables linked to resistance and resilience measured on the same function and on different ones were not necessarily the same. Despite fungi are also key soil microbes, with significant impact on ecosystems functions (Dighton, 2003, Six et al., 2006), in this forest ecosystem bacterial abundance was about 10 fold higher than fungal abundance (Figure 5.3 and 5.4). Therefore this study focused on bacterial communities and fungal diversity was not investigated. Nonetheless fungal abundance was included in the model-based approach used to determine the contribution of the variables measured to stability.
and it was not identified as significant predictor of resistance and resilience to disturbance of any of the soil processes investigated.

5.4.3. Effects of forest management practices on soil functional stability

Land use and land management have been repeatedly pointed out as factors affecting stability and therefore sustainability of soil environments (e. g. Seybold et al., 1999, Griffiths and Philippot, 2013). This study provides a methodical evaluation of the effects of irrigation and fertilisation within a forest plantation of *Eucalyptus saligna* and of land-use change on soil resistance and resilience. The structural equation modelling results showed that forest management practices had both indirect and direct effects on the stability of soil functions but these effects differed in their occurrence and nature depending on the function considered. Indirect effects of fertilisation and irrigation were mainly produced on the community composition variables (i. e. PCA axes scores) and fungal:bacterial ratio. Fertilisation is well known to affect bacterial community structure by increasing the availability of nutrients (O'Donnell et al., 2001, Kennedy et al., 2004), and its effects on microbial communities have been reported also for forest soils (Frey et al., 2004). Because soil moisture was adjusted to the same level in all microcosms during the experiment, the irrigation influence on community composition and fungal:bacterial ratio is more likely due to an increase in soil pH that accompanied both irrigation treatments rather to changes in water availability. Soil pH has, in fact, a strong influence on composition and abundance of bacterial communities (Lauber et al., 2009, Rousk et al., 2010). A study analysing soils collected across a long-term liming experiment showed that the relative abundance of bacteria is positively influenced by pH, while fungal abundances are not (Rousk et al., 2010). Therefore an increase in soil pH
associated with the irrigation treatments might have caused an increase of bacterial abundances, resulting in the decrease of the fungal:bacterial ratio, since fungi are less sensitive to pH changes. This supports the negative effect of irrigation treatments observed on the fungal:bacterial ratio (Figure 5.11).

Direct effects of forest management practices on stability were evident only for the resilience of respiration (i.e. basal respiration and total substrates response). Functional resistance was not directly influenced by management. Similarly, in a recent study comparing soil stability associated to different land use (pasture, plantation forest, native forest) in different locations in New Zealand, soil functional resistance to freeze-thaw disturbance measured using carbon utilisation profiles was not linked to land use (Wakelin et al., 2014).

In this study, irrigation was not found to directly affect the resilience of basal respiration, but it was associated with higher resilience of utilisation of carbon sources. Fertilisation had the strongest direct effect on the resilience of basal respiration. Because it has been shown to increase soil respiration indirectly through stimulation of productivity and litter-fall inputs (Tyree et al., 2006) and directly by increasing the nutrient content of the litter material, which stimulates decomposition of soil organic matter (Paul and Clark, 1989), fertilisation might have conferred resilience to disturbance of respiration.

The model-based approach used for identifying the drivers of stability was unable to identify any significant predictor of the resistance of β-glucosidase activity and basal respiration. Therefore it was not possible to evaluate the direct and indirect effects of forest management practices on these responses. Nonetheless it is possible that forest management practices influenced the resistance of these functions via variables that
were not analysed in this study. For example, soil organic matter has been proposed to be of key importance in maintaining soil resistance (Gregory et al., 2009).

The presence of significant direct paths, where the correlation between predictors and response is not significant (e. g. resilience of total respiration), suggests that forest management practices were affecting the stability via other unmeasured variables.

5.5. Conclusions and future directions

Previous manipulative experiments investigating the biodiversity-stability relationship, did not find any clear effect of diversity on resistance and resilience of soil functions, concluding that soil biological stability mostly depends on physico-chemical properties of soil (Griffiths et al., 2008). By subjecting field soils to a heat disturbance, we demonstrated that microbial communities are important drivers of soil functional stability in a forest ecosystem. In fact, microbial diversity components and community composition had the highest explanatory power of resistance and resilience of the majority of the functions analysed, when compared with the explanatory power of abiotic properties. Forest management practices were found to have contrasting direct effects on functional stability, depending on the soil function considered. These results need to be confirmed expanding the range of soil functions, ecosystems and land management types to precisely define the role that microorganisms play in the sustainability of soil ecosystems.
CHAPTER 6

General discussion
The enormous research effort directed to identify the consequences of biodiversity loss on ecosystem functioning in the last two decades has led to the general consensus that biodiversity loss reduces most ecosystem functions and affects their stability (Cardinale et al., 2012). Most of the research on this topic focused on plant and macrofaunal communities, thus our understanding of the functional consequences of microbial diversity loss is less well developed. This thesis has focused on the relationship between microbial diversity and functioning and stability of soil ecosystems. Because microorganisms are the most diverse group of organisms, and they play a key role in ecosystem functioning such as biogeochemical cycling, understanding the functional consequences of a loss in microbial diversity is of paramount importance in order to predict the effects of global diversity change.

While there is evidence that microbial communities are sensitive to environmental change and perturbation, the importance of community composition and diversity for ecosystem function and stability is less clear (Allison and Martiny, 2008). The significance of biodiversity loss in microbial communities is challenged by the concept of functional redundancy, which predicts that the loss of species would not alter ecosystem functioning if different species have the same function in a given ecosystem (Wohl et al., 2004). Because microbial diversity is huge, a high functional redundancy is expected in terrestrial systems (Nannipieri et al., 2003). Nonetheless, a certain degree of functional singularity has been observed in microbial communities and functional redundancy has been proposed to be greater for broad functions carried out by a wide group of microorganisms, than for specialised functions performed by narrower groups of microorganisms (Schimel, 1995). Overall, results achieved by assembling artificial communities with different
diversity levels or indirectly manipulating bacterial diversity were controversial (Bell et al., 2009), and our understanding of the relationship between microbial diversity and functioning under field conditions is limited.

Significant progress about the link between diversity and functional stability has been achieved through studies targeting aboveground organisms, while this relationship remains largely unexplored for soil microorganisms (Balvanera et al., 2006). The most supported hypothesis behind a positive diversity-stability relationship is the insurance hypothesis (Yachi and Loreau, 1999), according to which high biodiversity buffers the functional shifts induced by environmental variations.

Two major aims were addressed in this thesis. The first was to investigate the nature of the relationship between microbial diversity and ecosystem functioning in soil environments, by applying ecological principles derived from plant ecology. The second aim was to examine the relevance of microbial diversity for functional stability to disturbance of soil ecosystems. These two aims were achieved by means of controlled microcosms experiments, in order to identify patterns and causal mechanisms without environmental interferences, and by means of field studies to understand the link between naturally occurring microbial populations and their functioning in the real world.

In the following section, the hypotheses formulated in the general introduction (chapter 1) will be addressed in order to highlight the main findings achieved in this thesis.
1. A decrease in microbial diversity has stronger effects on the performance and stability of narrow functions than of broad functions.

This hypothesis was investigated by using the dilution-to-extinction approach in a liquid microcosms study (chapter 2). The microbial community was extracted by a soil sample and inoculated in soil extract medium (SEM), made with the same soil the inoculum was extracted from. Serial dilutions were used to create a diversity gradient, which was confirmed by T-RFLP analysis. The relationship between diversity and functioning was assessed by evaluating the respiration and utilisation of C sources of different complexity by communities with different diversity levels. The current debate regarding biodiversity conservation has its main focus on macroorganisms and mostly ignores microbial diversity. This has foundation in the belief that there is a huge functional redundancy among microbial communities as a result of their vast diversity. In this work, bacterial diversity was correlated to functioning by linear relationships. In fact, a reduction in diversity led to a significant decrease in community respiration rates and utilisation of lignin, representative of aggregate broad functions and narrow functions, respectively. The shape of the BEF depended on the function considered: the strongest relationship was observed for lignin utilisation, while the slope of the diversity-community respiration relationship was lower. Universal functions, such as glucose utilisation in this study, did not correlate to diversity, supporting the redundancy hypothesis. These results provided the evidence that diversity loss in microbial communities can affect differentially the overall functioning of the ecosystem depending on the function analysed, supporting our hypothesis.

Stability to disturbance of microbial communities with decreasing diversity was evaluated by assessing resistance and resilience of utilisation of C sources of
different complexity to a transient heat disturbance (chapter 2). Microbial communities with lower diversity showed lower functional resistance and resilience to disturbance, providing the evidence that the loss of diversity in soil systems has the potential to affect their functional stability. The disturbance applied had different effects on the resistance of the community depending on the function considered. The decrease in diversity, in fact, affected the resistance to disturbance of community respiration, but it did not impair the resistance of lignin and glucose utilization, possibly explained by functional redundancy.

Overall, these results allowed us to conclude that both broad and specialised functions of microbial communities depend on their diversity. Therefore, it is important that the debate regarding biodiversity conservation explicitly considers microbial diversity.

In the study reported in chapter 2, I deliberately excluded the soil physical component in order to focus on how differences in the structure and performance of bacterial communities translate into differences in functioning and stability. Nonetheless, the physical structure of soil affects the flow of gases and liquids and provides a range of microscale habitats for soil organisms (Young and Crawford, 2004). There is in fact a link between soil particles aggregation and microbial activity (Crawford et al., 2011). Therefore, it is likely that physical and biological interactions are able to impact soil processes, influencing the relationship between microbial diversity and soil functioning. Findings from functional stability studies in soil suggested that soil functional resistance and resilience are governed by the physicochemical structure of soil through its effect on microbial community composition and microbial physiology (Kuan et al., 2007, Griffiths et al., 2008).
Future research in this area must take into account the role of biophysical properties in soil ecosystem functioning and stability to disturbance.

In the context of understanding more generally how biodiversity affects ecosystem functioning, this microcosm experiment confirmed that microbial communities represent the ideal model systems for studying questions in community ecology and for biodiversity-ecosystem functioning studies. In fact, microorganisms grow and reproduce fast, making possible to conduct experiments over many generations under controlled laboratory conditions. These rapid generation times allow the communities to arrive at the equilibrium conditions upon which many ecological models are based, and allow simultaneous investigations of evolutionary and ecological processes (Bell et al., 2009). Unlike with larger and longer-lived organisms, in microbial systems it is possible to examine directly evolutionary processes such as niche differentiation, and to investigate their importance in determining functioning.

2. The relationship between microbial communities and ecosystem functions is influenced by the environmental variables in natural systems.

This hypothesis was addressed by investigating the nature of the relationship between microbial diversity and functioning by means of two separate field studies carried out in an agricultural system (chapter 3) and in a forest ecosystem (chapter 4). The diversity and composition of soil bacterial communities were analysed by means of Illumina MiSeq sequencing of the 16S rRNA gene, and microbial functions involved in nutrients cycling were analysed by means of potential enzyme activity, basal respiration and substrate induced respiration in both systems. The relationship between microbial diversity and soil functioning was investigated by means of
stepwise multiple regressions examining the explanatory power of microbial community variables and environmental properties. The purpose was to analyse how microbial diversity influences soil functioning under natural environmental conditions, taking into account the influence of land use and land management.

In the pasture-cropping system, the main predictors of soil functioning were edaphic properties and no correlation between microbial diversity and community composition and soil functioning was detected (chapter 3), suggesting that the soil environment played a stronger role in influencing the functional properties than biotic components in these systems. Nonetheless, both microbial community and functional responses to pasture-cropping treatments were observed, but these responses were independent from each other, since no statistical correlation was found.

In contrast with chapter 3, carbon and nutrient cycling functions were found linked to microbial communities in forest soils, where functioning was predicted by parameters representing bacterial diversity and abundance (chapter 4). In particular, the relationships between bacterial richness and enzyme activity and substrate-induced respiration followed positive linear trends, thus supporting the hypothesis that changes in microbial diversity can affect soil ecosystem processes. A significant contribution of edaphic factors to soil processes was also observed, supporting the importance of soil properties in influencing functioning highlighted in chapter 3.

The model-based approach used here represented a useful tool for analysing the BEF relationship in field studies, accounting for independent contributions of biotic and abiotic factors to ecosystem functioning. The significant contributions of different microbial properties to ecosystem functioning that emerged from this study pointed
out that the extreme complexity of soil biodiversity needs to be considered in all its components for a complete understanding of soil functioning. Therefore, future research should explicitly include other components of soil microbial diversity, such as fungi, micro and meiofauna, in these BEF field studies.

The relationship between microbial community’s properties and functioning were not found consistent in the forest and agricultural ecosystems investigated. A possible cause of the lack of a direct relationship between microbial community variables and soil functioning in chapter 3 could be ascribed to agricultural practices. Land management can have a significant influence on the BEF in soil ecosystem through the homogenisation of the soil landscape and the modification of biogeochemical cycles. In fact, local soil biodiversity is strongly driven by spatial heterogeneity, and the diversity of microhabitats found within a single soil profile could be equivalent to that found aboveground within an entire ecosystem (Myers et al., 2000). The agricultural management involves practices that disturb and homogenise soil structure and micro-landscape, possibly reducing microbial diversity, resulting in a simplification of species interactions and disrupting its relationship with functioning. The shape and direction of the biodiversity-functioning relationship can depend critically on the environmental context (Cardinale et al., 2000, Fridley, 2002, Tylianakis et al., 2008). Opposite relationships between microbial community structure and enzymatic activities were observed in soils with the same edaphic characteristics but different land use. The relationship was positive in a permanent grassland and negative in a arable cropping plot (Riah-Anglet et al., 2015). This suggests that not only edaphic properties and microbial
diversity drive the nature of the BEF relationship, but also soil management history plays a key role in soil functioning.

Therefore, these results demonstrate that under natural conditions microbial diversity plays an important role in driving soil functions together with environmental parameters, but its importance can be challenged by disruptive land management practices. Despite this is proven for bacteria by this work, the role played by fungi and soil fauna in the functioning of these ecosystems remains to be investigated. In this thesis, a relationship between bacterial richness and functioning was identified by means of a manipulative experiment (chapter 2) and observation of natural soils (chapter 4). Bacterial OTU richness was correlated with rates of soil functions by positive linear relationships both in microcosms and in field soils. Therefore, the kind of manipulative approach used in this work resulted not only in an effective method for the manipulation of bacterial diversity in BEF relationship studies, as demonstrated by our T-RFLP results (chapter 2), but also a useful tool for gaining realistic insights into the relationship between bacterial diversity and soil processes, as confirmed by the comparable results obtained by field studies and a laboratory experiment. While communities resulting from experimental manipulations of diversity clearly differ from complex communities from natural environments, this does not diminish the value and potential of this kind of studies to disentangle the key mechanisms behind the relationship between diversity and ecosystem functioning.

Moreover, the shape of the BEF relationship in most experimental studies focusing on plants indicates that initial losses of species have relatively small impacts on ecosystem functions, but increasing losses cause accelerating rates of change.
(Cardinale et al., 2011). In this work, the BEF relationship followed a positive linear trend, indicating that losses of bacterial species would have proportional effects on soil functioning, contradicting the functional redundancy theory for key ecosystem functions. This is surprising considering the huge diversity of microbial communities and the general functions measured in these experiments.

3. Resistance and resilience to disturbance of soil functions are influenced by microbial community diversity and composition.

This hypothesis was addressed examining the functional stability of forest soils with naturally different levels of bacterial diversity following a transient heat disturbance (chapter 5). The resistance and resilience to disturbance was measured by means of indices calculated on the rates of soil processes involved in nutrients cycling. By analysing the independent contributions of microbial community properties and environmental factors to resistance and resilience, it was possible to assess the relevance of microbial diversity and community composition for the functional stability in the forest soils under investigation. As hypothesised, the functional stability was best explained by bacterial diversity and community composition. These results are, to a certain extent, comparable to the results obtained by dilution of a soil microbial community in liquid cultures (chapter 2). Although in chapter 2 no direct correlation was observed between the diversity and functional stability to heat disturbance as it was found in chapter 5, a decrease in resistance and resilience was observed in microcosms with lower diversity. However it has to be considered that two different heat disturbances were used in the two experiments (i.e. 50°C for the liquid and 60°C for the soil experiment). These were chosen after conducting experimental trials aiming to determine a temperature that was high enough to produce a shock for the microbial communities, but not too high to drastically impair
the functional recovery, both in soil and liquid medium (see Appendix A and section 5.2.3). Because of the different physical properties of the two media used, the results of the trials lead to the selection of two different temperature treatments. The purpose of these experiments was not the comparison of the effects of heat shock on the stability of bacterial communities in two environments, but the analysis of the relationship between diversity and stability by means of experimental manipulation of the diversity of a community on one hand, and by observation of a natural gradient of diversity on the other hand. These findings demonstrated the significance of microbial communities for the functional response of soils to environmental disturbance.

The use of a range of different disturbances in this kind of experiment, such as fire, freeze-thaw, dry-wet, might reveal different aspects of soil microbial community responses to stress, drawing a better scenario of consequences on ecosystem functioning and sustainability. Any given stressor will affect composition and abundance of the species in a system according to their susceptibility to that disturbance (Jonsson et al., 2002). Therefore, biodiversity loss caused by a perturbation will have effects on the overall ecosystem functioning that depend on the kind of the disturbance. The experiments presented in this thesis and most of the experiments investigating the effects of a loss of biodiversity on functioning and stability of ecosystems used a single stressor at a time, whereas natural systems can suffer from multiple stressors and repeated exposures. The significance of microbial diversity for soil functional stability might increase in an ecosystem subjected to multiple disturbances. It is therefore important to address the effects of multiple stresses on the diversity-stability relationship in future studies.
Microbial diversity and ecosystem functioning under a multifunctional perspective

The results of the experiments described in this thesis also highlighted the importance of considering multiple functions in BEF studies. In fact, the BEF shape depended on the function considered. For instance, we observed the strongest relationship for lignin utilisation, a more specialised function, followed by basal respiration, while glucose utilisation was characterised by functional redundancy (chapter 2). Focusing on only one function for the assessment of the diversity-functioning relationship would have led to over- or underestimate the functional redundancy of the system. Most of the BEF studies focused mainly on the relationship between biodiversity and single ecosystem functions. Other studies found that increasing the number of functions considered changes the shape of the relationship, leading to different scenarios of the consequences of a biodiversity loss. Hector and Bagchi (2007), investigating the importance of simultaneous aspects of biodiversity on multiple ecosystem functions in experimental grasslands, found that the number of species necessary to sustain ecosystem functioning increased with the number of processes considered. An analysis of empirical data sets from bacteria, seagrasses and plants studies (Gamfeldt et al., 2008) suggested that the effect of biodiversity in natural ecosystems may be much larger than currently thought if considered under a multifunction perspective. Hence, ecosystem functioning as a set of several functions is more sensitive to species loss than are single functions.

The higher probability of sustaining multiple ecosystem functions with increasing species richness has been proven also for microbes (He et al., 2009, Peter et al., 2011b). He and colleagues (2009) found that in natural soil ecosystem greater bacterial diversity is required for sustaining multiple ecosystem functions. In chapter
4, different soil functions were predicted by different microbial components, suggesting that focusing on a wider range of ecosystem functions has the potential of finding a higher number of correlations with microbial properties. Microorganisms are so highly diverse and drive such a wide variety of ecosystem processes that, analysing the effects of their diversity on a single function would mean ignoring the possibility that different species perform multiple processes, leading to an overestimation of the amount of functional redundancy and to an underestimation of the risks of biodiversity loss. Hence, the relationship between biodiversity and ecosystem multifunctionality needs to be taken into account for conservation and management purposes. In fact, even if the performance of individual functions can be insensitive to an initial species loss, overall functioning can be more susceptible. The biodiversity-ecosystem multifunctionality relationship in soil systems cannot leave aside soil fungi and fauna, given their importance in many soil processes and should be focus of future research.

Overall data presented in this thesis suggest that the functioning and stability of soil ecosystems are strongly related to bacterial diversity. Complex relationships between bacterial community diversity, function, and stability exist. Multiple aspects of diversity (richness, evenness) can affect general and specific functions and their resistance and resilience. Importantly, these studies suggest that there may be different responses of soil function to disturbance. For diversity-functioning and diversity-stability relations under environmental conditions, more research is needed to understand system-specific trends before it will be possible to determine which patterns, if any, are general across microbial systems.
Although the boundaries of sustainable losses of microbial diversity still need to be assessed, a loss of microbial diversity has the potential to affect the functioning of ecosystems and their sustainability over time. Because microorganisms play a fundamental role in biogeochemical cycles (Falkowski et al., 2008), changes in their diversity can have important consequences on the whole ecosystem. Based on work from this thesis, the microbial component of ecosystems need to be included in conservation and management policies of the environmental resources.
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- 221 -


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- 223 -


APPENDIX A: Selection of temperature and duration of heat shock applied to measure stability to disturbance

Soil sampling, preparation of soil extract medium (SEM), extraction and inoculum of the microbial community were carried out as reported in section 2.2., creating a series of liquid microcosms in 250 Erlenmeyer flasks containing 30 ml of SEM.

Two shock temperatures were tested, 50° and 70° C, and each of them for two different shock durations, 30 minutes and 2 hours, for a total of four treatments. Two replicates and two sterile controls were subjected to each treatment, and a room temperature control treatment was added to the experiment.

All the microcosms were pre-incubated in a shaking incubator at 25 °C for two days to allow growth. Before applying the temperature shock, 1-ml aliquot from each microcosm was plated on 1/10 strength TSA plates (tryptic soy agar, Scharlau, Spain), after carrying out serial dilutions up to $10^{-8}$ in phosphate-buffered saline (PBS). Colonies that developed on agar were counted after incubating the plates for 19 hours in the dark at 25 °C.

The respiration rates were measured by means of MicroResp™ method. Both colonies counting and respiration measurements were repeated immediately after, and 2, 3, 6 days after the heat shock treatments, to assess the resilience.

1. Cell abundances

All heat shock treatments had negative effects on cell abundances, with the highest decrease in colony forming units per millilitre (CFUs/ml) in the 70 °C for 2 hours shock (96.01% of change from control). The rest of the treatments showed comparable levels of resistance: 82.5% for the 50 °C for 30 minutes shock, 81.05% for 50 °C for 2 hours, and 79.12% for 70 °C for 30 minutes (Figure A1).
Figure A1. Cell abundances before and after applying the heat shock estimated by plate counting (CFUs). The error bars represent the standard error of the mean.

2. Respiration

The results obtained from MicroResp™ measurement showed an overall decrease in respiration rates in all treatments after the shock. In all the treatments, in fact, the respiration rate decreased abruptly immediately after the shock, and it increased again on the second and the third day, except for the 70 °C for 30 minutes treatment, which continued decreasing until the end of the experiment (Figure A2).
Figure A2. Respiration rates for the four treatments and the non-shocked control (RT). The red arrow indicates the occurrence of the heat shocks, and the horizontal axis the measurement points: BS=before shock, AS=after shock, day 2, day 3, day 6 indicates respectively the 2<sup>nd</sup>, the 3<sup>rd</sup>, and the 6<sup>th</sup> day after the shock. The error bars indicate the standard error of the mean.

None of the shocked microcosms returned to respiration rates similar to those observed before the disturbance, except for those subjected to 50 °C for 30 minutes. Both the 70 °C treatments show the lowest respiration rates after shock for the all duration of the experiment.

Figure A3 shows the functional resistance to disturbance of the microbial communities cultured during this experiment. The communities subjected to 30 minutes shocks at both temperatures shows higher resistance to disturbance (59.39% change for 50 °C and 66.8% for 70 °C), compared to the 2 hours treatments (86.65% and 103.52% respectively).
Figure A3. Respiration rates for the four treatments before and immediately after heat shocks. The line represents the resistance to disturbance expressed as percentage of change between before and after the shock. Error bars represents the standard error of the mean.

Microbial communities subjected to 70 °C shock had the lowest respiration rates. The 70 °C for 2 hours treatments also resulted in the lowest resistance (highest % change) of communities, both from functional and cell abundances perspectives. On the other hand, the communities subjected to 50 °C for 2 hours shock show higher resistance both from cell abundances and functional point view, and seem to recover more from disturbance. Despite this, the communities are not resilient to this treatment, because they are unable to recover to before shock functions. In this experiment, it is possible to observe resilience of functioning only in the microcosms subject to 50 °C for 30 minutes. Moreover, this treatment also results in the most resistant microbial communities, as supported by results obtained from both respiration rates and plate counting. Therefore this treatment was considered suitable for future stability experiments because it allows the microbial community to fully recover after disturbance. In fact, without having the communities with non-
manipulated diversity recovering after stress, it would not be possible to assess the resilience of treatments where the diversity is reduced.

Moreover, it is possible to observe that resistance measured by means of functional parameters, such as community respiration (i.e. functional resistance), has higher resolution and precision compared to the resistance of cell abundances. In fact, while in Figure A there is nearly no difference between the first three treatments, the functional resistance values clearly point out that communities subjected to 2 hours heat shocks are less resistant than those that underwent treatments of 30 minutes.
APPENDIX B: Biomass recovery in soil extract medium experiments

**Figure B1.** Bacterial biomass recovery at different dilution levels measured by means of qPCR of 16S rRNA gene during separate experiments with different pre-incubation times. Figure A and B show data from two experiments carried out with soil extract medium and microbial community extracted from a cotton farm’s soil located in Narrabri (NSW, Australia), with a pre-incubation time of three days. Figure C show the bacterial recovery after four days during an experiment carried out with soil extract medium and microbial community extracted from a sugar cane plantation soil located in Innisfail (QLD, Australia).
APPENDIX C: Optimisation of substrate concentration for potential enzyme assays at Moora field site

Figure C1. Substrate saturation curve for the activity of β-glucosidase (A), N-acetyl-glucosaminidase (B), and acid phosphatase (C) in soils from Moora experiment. Soils from the adjacent untreated plot was used for this optimisation trial. Different symbols indicate different soil subsamples.
APPENDIX D: Optimisation of substrate concentration for potential enzyme assays at HFE experiment

Figure D1 Substrate saturation curve for the activity of β-glucosidase (A), N-acetyl-glucosaminidase (B), and acid phosphatase (C) in soils from HFE and the nearby natural forest. IF = irrigation + fertilisation; C = control; F = fertilisation; I = irrigation; NW = natural woodland.
APPENDIX E: Functional measurements used for the calculation of resistance and resilience indexes

1. Potential enzyme activity

![Graph A](image1)

**Figure E1.** Potential enzyme activity of β-glucosidase (A), N-acetyl-glucosaminidase (C), and acid phosphatase (C) measured one day after the disturbance was applied, in both room temperature control and perturbed soils for the calculation of resistance indexes. I = irrigation; F = fertilisation; IF = irrigation + fertilisation; C = control; NF = natural forest. Error bars indicate ± one standard error (n=8 for NF, n=4 for the others).
Figure E2. Potential enzyme activity of β-glucosidase (A), N-acetyl-glucosaminidase (C), and acid phosphatase (C) measured one month after the disturbance was applied, in both room temperature control and perturbed soils, for the calculation of resilience indexes. I = irrigation; F = fertilisation; IF = irrigation + fertilisation; C = control; NF = natural forest. Error bars indicate ± one standard error (n=8 for NF, n=4 for the others).
2. Soil respiration

![Graph A](image1)

![Graph B](image2)

**Figure E3** Mean respiration responses measured two days after heat disturbance in the control and perturbed soils for the calculation of resistance indexes of basal respiration (A) and total respiration responses to carbon substrates (B). I = irrigation; F = fertilisation; IF = irrigation + fertilisation; C = control; NF = natural forest. Error bars indicate ± one standard error (n=8 for NF, n=4 for the others).
Figure E4 Mean respiration responses measured one month after heat disturbance in the control and perturbed soils for the calculation of resilience indexes of basal respiration (A) and total respiration responses to carbon substrates (B). I = irrigation; F = fertilisation; IF = irrigation + fertilisation; C = control; NF = natural forest. Error bars indicate ± one standard error (n=8 for NF, n=4 for the others).