Flinders University

# Harnessing the soil microbiome to improve ecosystem restoration in a global biodiversity hotspot

By

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BSc (Hons)

*Thesis*
*Submitted to Flinders University*
*for the degree of*

**Doctor of Philosophy (PhD)**
College of Science and Engineering
29th of April 2025

*Remnant mallee heath vegetation on Yarraweyah Falls Reserve, Noongar Country, Western Australia*

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# Thesis Abstract

The effective restoration of degraded ecosystems is essential in addressing the twin global crises of biodiversity decline and climate change. However, restoration success is elusive, and the development of innovative methods is needed to address these shortcomings. Soil – our planet's most biodiverse habitat – is home to at least 60% of Earth's species yet it is inadequately integrated into the practice and science of restoration. The soil microbiome is a crucial component of healthy ecosystems. Improved integration of the soil microbiome into the restoration of biodiverse, functional and resilient ecosystems has the potential to improve restoration success and help address the global crises of biodiversity decline and climate change.

In this thesis, I take on exploring current and emerging practical uses of the soil microbiome in the restoration of functional and resilient ecosystems in southwest Western Australia – a global biodiversity hotspot. In chapter one, I present a comprehensive forward-looking review that covers current knowledge and future directions for the practical application of soil microbiota to improve ecosystem restoration (published in *Biological Reviews*). The review highlights how soil microbiota are currently integrated in ecosystem restoration, identifies knowledge gaps constraining their integration and suggests research to address these knowledge gaps. In chapter two, I use amplicon sequencing techniques in an observational study to assess the state of recovery of soil bacterial communities following landscape-scale revegetation across six restoration sites in southwest Western Australia (published in *Biological Conservation*). I identify key persistent agricultural land-use legacies that have inhibited the recovery of these important ecological communities. In chapter three, I use shotgun metagenomic sequencing

approaches to explore how expanding from taxonomic-based metrics by examining functional potential can provide a more ecologically informative picture of the recovery of specific microbial-mediated ecosystem functions. In this chapter, I show that both taxonomic and functional gene compositions have not entirely recovered following restoration efforts and land-use legacies associate with these altered compositions. While results may indicate incomplete recovery, they do not necessarily mean dysfunction but likely reflect functional adaptations to altered conditions. In chapter four, I embed a soil translocation experiment into active restoration sites in southwest Western Australia to assess the effectiveness of three different soil translocation methods on the establishment of beneficial soil microbiota. I show that retaining soil structural integrity through intact soil translocations is important in achieving successful inoculation. By contrast, mixed soil translocations and surface spreading – the predominant method of soil translocation – saw microbial communities diverge away from the microbial profile of donor sites. Together, these thesis components employ novel methods to identify, address and close knowledge gaps towards improving ecosystem restoration outcomes through improved integration of the soil microbiome.

# Declaration

I certify that this thesis:

1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university
2. and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University and
3. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Shawn D. Peddle

28th of March 2025

# List of Supervisors

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# Acknowledgements

Firstly, I acknowledge all of the traditional owners of the lands upon which I live, work and study: The Kaurna People, and the Goreng and Minang People of the Noongar Nation. I pay my respects to their Elders past, present and emerging.

I would like to acknowledge the dedication, support and leadership of my primary supervisor, Associate Prof. Martin Breed. Martin's outstanding supervision has not wavered throughout my candidature. His knack in striving for excellence without overreaching has been instrumental to my development as a researcher, the success of my projects, and my completion. Martin's support throughout this journey exceeded the requirements of a supervisor. Thank you, Martin. I extend the same acknowledgements to my associate supervisors, Dr Craig Liddicoat and Prof. Robert Edwards, and my adjunct supervisor, Dr Siegfried Krauss. I am grateful to each of them for their guidance, direction and patience at all steps of my candidature.

Tremendous gratitude also goes to my colleagues and peers within the FORE Lab and the wider Flinders University community who have made substantial contributions along the way. Thanks to Christian Cando-Dumancela, Riley Hodgson, Carl Watson, Tarryn Davies, Nicole Fickling, Alex Taylor, Dr Joel Brame, Dr Jake Robinson, and the many others who listened to – and helped solve – my problems throughout the journey. Thanks to Kieren Beaumont and Sunita Ramesh for your role as internal assessors in ensuring my projects were ambitious yet achievable. Thanks to those the FAME Lab, Prof. Rob Edwards, Prof. Liz Dinsdale, Dr Michael Doane, Dr Michael Roach and others whose bioinformatics wizardry (and funding support!) helped me overcome a significant hurdle in my journey.

I am grateful for the funding support I have received for my research. Thank you to Bill Holsworth and the Holsworth Wildlife Research Endowment through the Ecological Society of Australia (ESA) for your generous support of my (and hundreds of others) PhD research. Thank you to Peter and Maxine Wilshaw for your generous support of my research through Bush Heritage Australia. I feel better about the world knowing there are people like Mr Holsworth and Mr and Mrs Wilshaw who are willing to contribute what they can to help address some of the impacts we have had on our planet. Again, thank you for this.

I am also thankful to have been a recipient of the Australian Government Research Training Program Scholarship.

Thank you to Bush Heritage Australia (BHA) for their dedication to improving conservation and restoration outcomes around Australia, and for all the support and opportunity they have provided throughout. Special thanks to Michelle Hall, Jaclyn Smith, Angela Sanders, Alex Hams and many others at BHA.

Lastly, but probably most importantly, thank you to my wife and family. I had the immense benefit of proceeding on my PhD journey about three years behind my wife Kristen’s own PhD journey. The intricacies I learnt second-hand through watching and hearing about her successes and challenges gave me an inside edge and helped me immensely in my own path to completion. Her support, love, care, empathy and friendship throughout all the challenges we faced together over the last 4 years where the most important factors in getting me to where I am now. I would

be remiss if did not mention old Rogue, and his understudies Moose and Grizz, for all they bring to our lives too. And thank you to my parents and family back in Canada, my ‘gap year’ may have blown out to more than 20, but I got there in the end!

## Details of co-authorship

Here I provide details of the contributions of co-authorship for each of my thesis chapters:

**Chapter one: Practical applications of soil microbiota to improve ecosystem restoration: current knowledge and future directions**

**Peddle, S.D.**, Hodgson, R.J., Borrett, R.J., Brachmann, S., Davies, T.C., Erickson, T.E., Liddicoat, C., Muñoz-Rojas, M., Robinson, J.M., Watson, C.D., Krauss, S.L. and Breed, M.F.

The candidate was the primary author of the manuscript, conducted the majority of the work, and led all aspects of the project. Conceptualisation and project design: **SDP**, RJH, RJB, CDW, SLK, MFB. Manuscript writing – original draft: **SDP**, RJH, MFB. All authors contributed to revisions and editing and approved the final submission. This chapter has been published in *Biological Reviews*. https://doi.org/10.1111/brv.13124

**Chapter two: Agricultural land-use legacies affect soil bacterial communities following restoration in a global biodiversity hotspot**

**Peddle, S.D.**, Cando-Dumancela, C., Krauss, S.L., Liddicoat, C., Sanders, A. and Breed, M.F.

The candidate was the primary author of the manuscript, conducted the majority of the work, and led all aspects of the project. Conceptualisation and project design: **SDP**, SLK, AS, MFB. Field work and data generation: **SDP**, CCD, AS. Formal analysis: **SDP**, CL, MFB. Manuscript writing – original draft: **SDP**. All authors contributed to revisions and editing and approved the final submission. This chapter has been published in *Biological Conservation*.
https://doi.org/10.1016/j.biocon.2023.110437

**Chapter three: Soil microbial functions associate with persistent agricultural legacies and indicate an alternative stable state following restoration plantings in a global biodiversity hotspot**

**Peddle, S.D.**, Cando-Dumancela, C., Costin, S., Davies, T., Doane, M.P., Edwards, R.A., Hodgson, R.J., Krauss, S.L., Liddicoat, C. and Breed, M.F.

The candidate was the primary author of the manuscript, conducted the majority of the work, and led all aspects of the project. Conceptualisation and project design: **SDP**, SLK, MFB. Field work and data generation: **SDP**, CCD. Formal analysis: **SDP**, RAE, CL, MFB. Manuscript writing – original draft: **SDP**. All authors contributed to revisions and editing and approved the inclusion of this chapter in my thesis. This chapter has been formatted for submission to *Restoration Ecology* and is available as a preprint on Authorea: https://doi.org/10.22541/au.174301721.10879091/v1

**Chapter four: Stronger together: intact soil translocation increases the resilience of inoculated microbial communities**

**Peddle, S.D.**, Cando-Dumancela, C., Davies, T., Edwards, R.A., Hodgson, R.J., Krauss, S.L., Liddicoat, C., Sanders, A. and Breed, M.F.

The candidate was the primary author of the manuscript, conducted the majority of the work, and led all aspects of the project. Conceptualisation and project design: **SDP**, SLK, MFB. Field work and data generation: **SDP**, CCD, SLK. Formal analysis: **SDP**, CL. Manuscript writing – original draft: **SDP**. All authors contributed to revisions and editing and approved the inclusion of this chapter in my thesis. This chapter has been formatted for submission to *Ecology Letters* and is available as a preprint on Authorea: https://doi.org/10.22541/au.174180429.99271478/v1

# List of published works

Here I provide a list of all published works I have contributed to during my candidature:

Hodgson, R.J., Liddicoat, C., Cando-Dumancela, C., Fickling, N.W., **Peddle, S.D.**, Ramesh, S. and Breed, M.F., 2024. Increasing aridity strengthens the core bacterial rhizosphere associations in the pan-palaeotropical C4 grass, Themeda triandra. *Applied Soil Ecology*. https://doi.org/10.1016/j.apsoil.2024.105514

**Peddle, S.D.**, Hodgson, R.J., Borrett, R.J., Brachmann, S., Davies, T.C., Erickson, T.E., Liddicoat, C., Muñoz-Rojas, M., Robinson, J.M., Watson, C.D., Krauss, S.L. and Breed, M.F., 2024. Practical applications of soil microbiota to improve ecosystem restoration: current knowledge and future directions. *Biological Reviews*. https://doi.org/10.1111/brv.13124

**Peddle, S.D.**, Cando-Dumancela, C., Krauss, S.L., Liddicoat, C., Sanders, A. and Breed, M.F., 2024. Agricultural land-use legacies affect soil bacterial communities following restoration in a global biodiversity hotspot. *Biological Conservation*. https://doi.org/10.1016/j.biocon.2023.110437

Birnbaum, C., Dearnaley, J., Egidi, E., Frew, A., Hopkins, A., Powell, J., Aguilar-Trigueros, C., Liddicoat, C., Albornoz, F., Heuck, M. K., Dadzie, F. A., Florence, L., Singh, P., Mansfield, T., Rajapaksha, K., Stewart, J., Rallo, P., **Peddle, S. D.**, and Chiarenza, G., 2023. Integrating soil microbial communities into fundamental ecology, conservation, and restoration: examples from Australia. *New Phytologist*.

https://doi.org/10.1111/nph.19440

Robinson, J.M., Hodgson, R., Krauss, S.L., Liddicoat, C., Malik, A.A., Martin, B.C., Mohr, J.J., Moreno-Mateos, D., Muñoz-Rojas, M., **Peddle, S.D.,** and Breed, M.F., 2023. Opportunities and challenges for microbiomics in ecosystem restoration. *Trends in Ecology & Evolution*. https://doi.org/10.1016/j.tree.2023.07.009

**Peddle, S.D.**, Bissett, A., Borrett, R.J., Bullock, P., Gardner, M.G., Liddicoat, C., Tibbett, M., Breed, M.F. and Krauss, S.L., 2023. Soil DNA chronosequence analysis shows bacterial community re-assembly following post-mining forest rehabilitation. *Restoration Ecology.* https://doi.org/10.1111/rec.13706

Cando-Dumancela, C., Davies, T., Hodgson, R.J., Liddicoat, C., **Peddle, S.D.**, Watson, C.D. and Breed, M.F., 2023. A practical guide for restoration ecologists to manage microbial contamination risks before laboratory processes during microbiota restoration studies. *Restoration Ecology*. https://doi.org/10.1111/rec.13687

Lem, A.J., Liddicoat, C., Bissett, A., Cando-Dumancela, C., Gardner, M.G., **Peddle, S.D.**, Watson, C.D. and Breed, M.F., 2022. Does revegetation cause soil microbiota recovery? Evidence from revisiting a revegetation chronosequence 6 years after initial sampling. *Restoration Ecology*. https://doi.org/10.1111/rec.13635

Watson, C.D., Gardner, M.G., Hodgson, R.J., Liddicoat, C., **Peddle, S.D.** and Breed, M.F., 2022. Global meta-analysis shows progress towards recovery of soil microbiota following revegetation. *Biological Conservation*. https://doi.org/10.1016/j.biocon.2022.109592

Liddicoat, C., Krauss, S.L., Bissett, A., Borrett, R.J., Ducki, L.C., **Peddle, S.D.**, Bullock, P., Dobrowolski, M.P., Grigg, A., Tibbett, M. and Breed, M.F., 2022. Next generation restoration metrics: Using soil eDNA bacterial community data to measure trajectories towards rehabilitation targets. *Journal of Environmental Management*. https://doi.org/10.1016/j.jenvman.2022.114748

## Thesis structure

This thesis is structured in adherence to the HDR Thesis Rules as set by Flinders University. The thesis can be easily navigated through the Table of Contents.

The thesis contains four primary chapters: a comprehensive review and three data chapters. The primary chapters are preceded by a thesis summary and a general introduction and succeeded by a general discussion. Both the general introduction and general discussion are kept relatively brief as each chapter contains important introduction and discussion materials.

Chapters one and two have been peer-reviewed and published in *Biological Reviews* and *Biological Conservation*. They are reproduced here under a CC BY 4.0 open access licence (http://creativecommons.org/licenses/by/4.0/).

Although both journals have their own unique formatting and referencing requirements, this thesis shares a single format and referencing style throughout. Figures have been embedded within the text after their first mention. I have chosen to retain some journal-specific formatting within the published chapters where it maintains the authenticity of the published version. For example, section headings in chapter 1 are identified with Roman numerals whereas section headings in chapter 2 are identified with Arabic numerals. The thesis contains a single reference list for all chapters following the main text. Supplementary information from each chapter is included separately as Appendices.

# General introduction

## Background

The effective restoration of degraded ecosystems must be rapidly scaled-up to unprecedented levels in order to address the existential threats of the global biodiversity and climate crises (Silliman *et al.*, 2024). Accordingly, the United Nations has declared 2021 – 2030 *The Decade on Ecosystem Restoration* with the aim to scale-up and unlock restoration implementation potential (Waltham *et al.*, 2020). While the science and practice of ecosystem restoration – defined as *"the process of assisting the recovery of an ecosystem that has been degraded, damaged or destroyed"* (Gann *et al.*, 2019a) – has been advanced substantially over the past few decades, many restoration efforts still do not achieve their goals and failure is widely underreported (Rillig *et al.*, 2024; Hobbs, 2009; Prober *et al.*, 2025). Ecosystem restoration has previously been concentrated on aboveground ecosystem attributes (e.g., revegetation, animal reintroductions). However, as soil is home to somewhere between 60% (Anthony, Bender & van der Heijden, 2023) and 99.9% of the earth's species (Blakemore, 2025), increased attention is being given to the importance of soil as a foundation for the majority of ecosystem processes (Nannipieri *et al.*, 2017; Raupp, Carrillo & Nielsen, 2024). As such, advancements in improving the integration of belowground ecological communities into restoration are crucial to help address persistent restoration shortfalls.

Soil microbiomes – the communities of bacteria, archaea, fungi, viruses, and their internal and external structural elements in soil (Berg *et al.*, 2020) – play key roles in soil structural formation, nutrient cycling, plant productivity and carbon sequestration.

The soil microbiome is linked with aboveground ecosystem components at species, community, and ecosystem levels (Fierer, 2017; Heneghan *et al.*, 2008). As land use and environmental changes impact on one ecosystem component, they can lead to up or downstream shifts in the other. Therefore, improving our understanding of both the response of soil microbiota to other restoration interventions (e.g., restoration plantings) as well as how to directly target the restoration of soil microbiota is crucial to effectively return biodiverse and functional ecosystems.

Microbial distributions are shaped by a complex interplay of ecological and environmental factors (Liu & Salles, 2024; Martiny *et al.*, 2006). A long-held but still disputed principle in microbial biogeography is the Bass-Becking hypothesis, which states that *"everything is everywhere, but, the environment selects"* (Baas-Becking, 1934). This idea suggests that microbial taxa have broad dispersal capacities, but their local composition is primarily determined by environmental conditions. In the context of ecosystem restoration, soil microbial communities are influenced not only by present-day environmental factors but also by historical land-use legacies, such as those associated with prior agricultural disturbance (Jangid *et al.*, 2011; Osburn, Aylward & Barrett, 2021). These legacies can alter soil properties in ways that shape microbial community recovery and reinforce alternative stable states that differ from undisturbed ecosystems. While this thesis explores the relationship between microbial composition and soil abiotic properties in restored landscapes, it does not explicitly disentangle the relative contributions of environmental filtering, dispersal limitation, or historical contingencies. Nevertheless, the findings provide insight into the extent to which environmental selection may drive microbial community assembly following land-use change and ecosystem restoration.

Biodiversity of Western Australia's Southwest floristic region has been severely impacted by historic land clearing and subsequent land use conversions for agriculture. These impacts have resulted in the area being listed as a global biodiversity hotspot, an area with exceptional concentrations of endemic species undergoing exceptional loss of habitat (Myers *et al.*, 2000). As of 2017, the southwest floristic region contained 8,379 described native vascular plant species, 47% of which are endemic and 28% of which are of conservation concern at the state level (Gioia & Hopper, 2017). The high levels of degradation in large areas of this region have caused concerns that the possibility of restoration to historic conditions may be unlikely (Hopper, 2009). However, others have argued that continued advancements to, and investments in, restoration practice and science are warranted to ensure the conservation and restoration of the region's crucial biodiversity (Cramer, Hobbs & Standish, 2008; Standish & Hobbs, 2010).

In line with the calls to advance restoration progress, multiple conservation-focussed organisations are now engaged in restoring connectivity and functionality in the region. The largest of these restoration projects in the region – Gondwana Link – aims to restore landscape connectivity across more than 1,000 km of land between the remnant forests of the far southwest corner of Western Australia and the Nullarbor Plains to the northeast (Bradby, Keesing & Wardell‐Johnson, 2016). Bush Heritage Australia (BHA) – one organisation involved in Gondwana Link – has been acquiring and actively revegetating post-agricultural properties in southwest Western Australia's Fitz-Stirling region with the aim of restoring functional native ecological communities. BHA currently own, manage or are involved in restoration/conservation

work on lands spanning approximately 7,000 hectares in the Fitz-Stirling region and have recorded over 1,000 native taxa on their reserves (Figure 1) (Bush Heritage Australia, 2023). Historic agricultural practices in the region – including long-term fertiliser application in a region where vegetation is adapted to nutrient poor soil conditions – are well known to severely impact both abiotic and biotic soil properties including soil nutrient levels and soil microbiota (Standish *et al.*, 2006). These disturbances and their post-agricultural legacies can impede the effective restoration of functional and biodiverse native ecosystems (Parkhurst *et al.*, 2022a). Accordingly, novel considerations and advancements towards overcoming post-agricultural restoration barriers will be integral to restoring functional native ecosystems both in southwest Western Australia and the wider world.

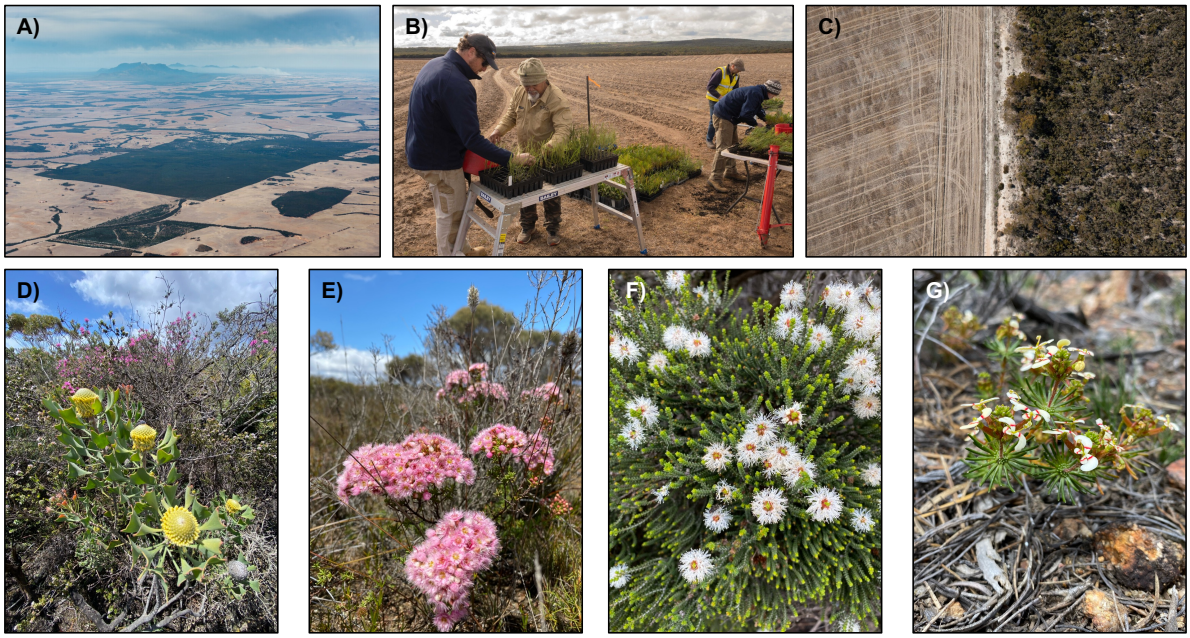


***Figure 1*** *Photographs from Bush Heritage Australia reserves in the Fitz-Stirling region of southwest Western Australia. (A) Aerial photo of Red Moort Reserve with the Stirling Ranges in the background showing the degree of habitat fragmentation in the region [photo: Greenskills, reproduced with permission]; (B) Restoration planting work on a new BHA reserve, Ediegarrup [photo: Adrian Gaspari, reproduced with*

*permission]; (C) Aerial photo showing the contrast between post-agricultural and remnant land [photo: Grassland Films, reproduced with permission]; (D-G) Photos of a variety of endemic plant species on Monjebup reserve [photos: Shawn Peddle].*

**Thesis aims and outline**

My PhD research is embedded within ongoing restoration projects led by BHA in Western Australia's southwest Australian floristic region, a global biodiversity hotspot (Myers *et al.*, 2000). My thesis is focussed on addressing knowledge gaps regarding the responses of soil microbial communities and their functional capacities following post-agricultural restoration plantings in a highly biodiverse mallee heath vegetation community. Additionally, the thesis explores a novel method of directly targeting the restoration of soil microbial communities through the translocation of soil with the intention of inoculating whole microbial communities. All four thesis chapters are focused on how the inclusion and consideration of the soil microbiome can improve ecosystem restoration outcomes.

In this thesis I aim to identify and address knowledge gaps hindering the effective integration of the soil microbiome into ecosystem restoration. Each of my PhD chapters address this broad aim. Here, I outline each chapter's aims, major conclusions and contributions to restoration science and practice:

**Chapter 1: Practical applications of soil microbiota to improve ecosystem restoration: current knowledge and future directions**

**(**Published in *Biological Reviews*: https://doi.org/10.1111/brv.13124)

In this chapter I aim to provide a thorough and forward-looking review that examines existing knowledge and future prospects for leveraging soil microbiota in ecosystem restoration. This review explores current applications of soil microbiota in restoration efforts, identifies key knowledge gaps limiting their integration, and proposes research strategies to bridge these gaps. I outline practical applications across restoration planning, direct interventions, and monitoring strategies, emphasising the need to integrate and consider soil microbiota into restoration targets (figure 2). I demonstrate how embedding microbiota-focused experiments in restoration projects, alongside statistical modelling approaches, can improve causal understanding and guide research priorities. I identify the inoculation of soil microbiota as a promising but underutilised strategy, with knowledge gaps surrounding establishment success and effective methods. Additionally, I show how microbial diversity, composition, and function can serve as indicators of restoration progress. I provide a framework for applying soil microbiota in ecosystem restoration and emphasise the need for researcher-practitioner collaboration to bridge knowledge gaps and enhance restoration outcomes. Importantly, while this review identifies numerous knowledge gaps inhibiting effective integration of microbiota into restoration, only a few are addressed in the succeeding thesis chapters.

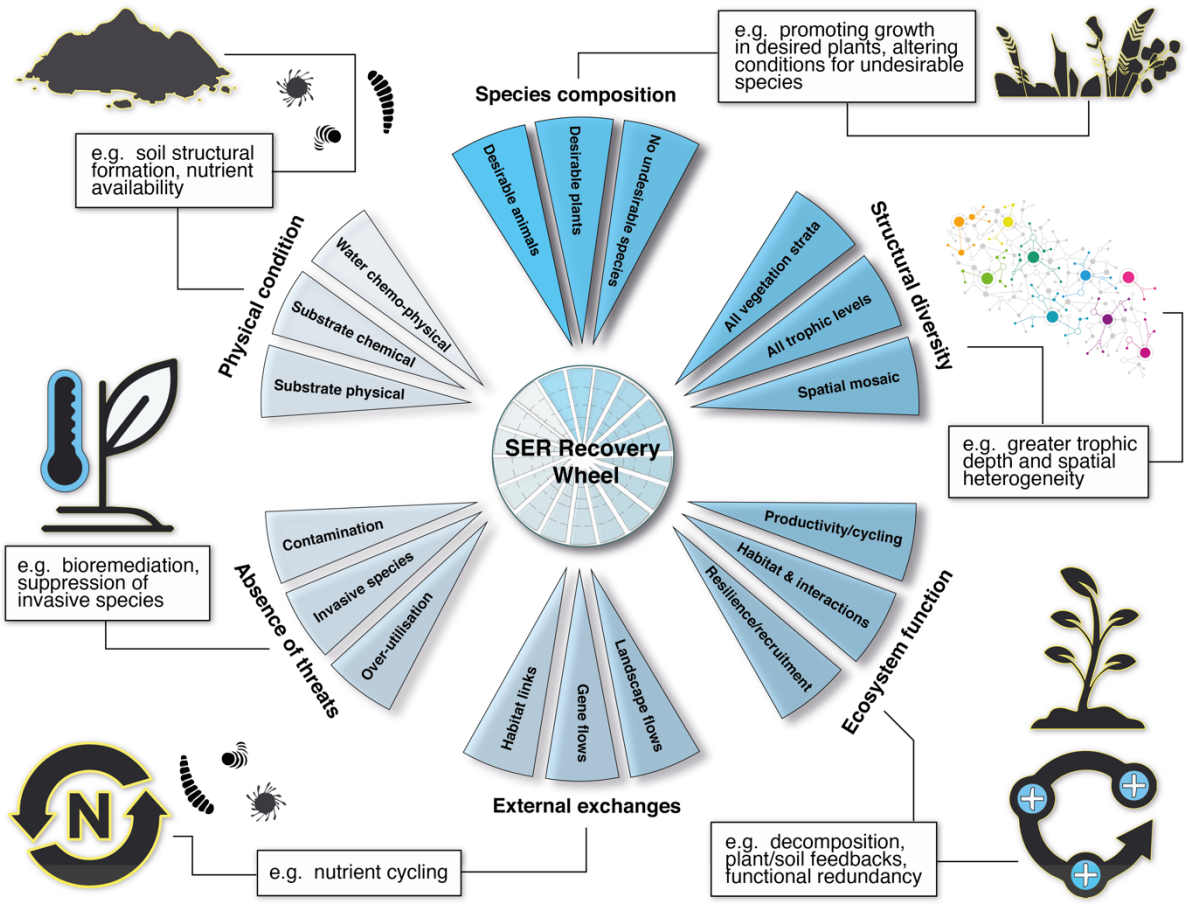


***Figure 2*** *The Society for Ecological Restoration (SER) recovery wheel (Gann et al., 2019) and how improved integration of soil microbiota into the planning, intervention, and monitoring phases of ecosystem restoration projects could contribute to each of the six recovery outcome themes (*Peddle *et al.* 2024, page 3).

## Chapter 2: Agricultural land-use legacies affect soil bacterial communities following restoration in a global biodiversity hotspot

(Published in *Biological Conservation*: https://doi.org/10.1016/j.biocon.2023.110437]

Monitoring soil microbial communities following restoration can provide important context on post restoration ecosystem recovery. In this data chapter I aim to assess the recovery of soil bacterial communities following post-agricultural restoration plantings across six restoration sites in southwest Western Australia (figure 3) using

amplicon sequencing of bacterial 16S rRNA genes. I show that soil bacterial communities in revegetated and degraded sites remain similar to one another but are distinctly different to those in remnant bushland communities, even up to 17 years after restoration. I identify elevated soil phosphorus as a key factor limiting microbial recovery, highlighting the lasting impact of agricultural land-use legacies on soil microbiota. These findings underscore a major challenge for conservation and restoration practitioners seeking to integrate soil microbiota into restoration efforts in ancient, nutrient-poor landscapes, where soil nutrient legacies may hinder microbial community reassembly.


***Figure 3*** *Photographs of a (A) degraded land condition site at Monjebup Reserve, (B) revegetated land condition site at Monjebup Reserve, and (C) remnant land condition site at Yarraweyah Falls Reserve.*

**Chapter 3 Soil microbial functions associate with persistent agricultural legacies and indicate an alternative stable state following restoration plantings in a global biodiversity hotspot**

(Prepared to submit to *Restoration Ecology*, preprint available via *Authorea* at: https://doi.org/10.22541/au.174301721.10879091/v1)

Assessing microbial taxonomic diversity and composition is increasingly used to gauge restoration success. However, due to high functional redundancy and weak

taxa-function links in soil, taxonomic data alone may be insufficient to infer ecological recovery. In this chapter, I assess the recovery of microbial functional potential following post-agricultural restoration in southwest Western Australia using shotgun metagenomic sequencing. I show that while the overall number of microbial functions does not differ between land conditions, functional compositions remain distinct between revegetated and remnant sites, with strong associations to soil abiotic properties, including phosphorus. Despite a general lack of functional recovery, I conclude that this likely does not represent a dysfunctional system but a functional response to an alternative stable state driven by soil abiotic conditions.

**Chapter four: Stronger together: intact soil translocation increases the resilience of inoculated microbial communities**

(Prepared to submit to *Ecology Letters*, preprint available via *Authorea,* DOI: https://doi.org/10.22541/au.174180429.99271478/v1)

My previous data chapters both focussed on post-restoration observational monitoring to assess recovery of microbial communities and their functions. In this chapter, I embedded an experiment into two post-agricultural restoration sites in southwest Western Australia to assess the efficacy of three soil translocation methods for inoculating whole microbial communities (Figure 4). I demonstrate that retaining soil structural integrity through intact translocations results in the improved establishment of donor-site microbial communities. Surface spreading— the most widely used method of soil translocation in restoration—resulted in microbial communities diverging away from the donor compositions and becoming more like recipient site communities. These findings suggest a need for the restoration sector

to reconsider current microbial inoculation approaches and highlight the potential benefits of intact soil translocations. To enhance ecosystem recovery, greater investment and innovation are needed to scale up effective soil translocation methods.

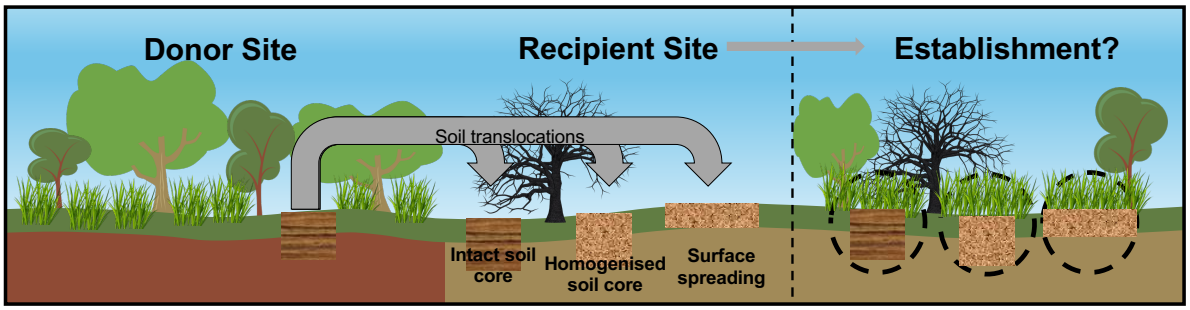


***Figure 4*** *Graphical illustration of the concept of testing the efficacy of three soil translocation treatments (intact core, mixed core and surface spreading) for successfully establishing soil microbial communities.*

Following my primary thesis chapters, I conclude with a general discussion of the implications of the thesis results for the practice and science of ecosystem restoration, their novel contributions to the field, and future research directions needed to continue to advance the integration of the soil microbiome towards improving ecosystem restoration.

# Chapter 1: Practical applications of soil microbiota to improve ecosystem restoration: current knowledge and future directions

Shawn D. Peddle[1,*], Riley J. Hodgson[1], Ryan J. Borrett[2], Stella Brachmann[3], Tarryn C. Davies[1], Todd E. Erickson[4,5], Craig Liddicoat[1], Miriam Muñoz-Rojas[6,7], Jake M. Robinson[1], Carl D. Watson[1], Siegfried L. Krauss[4,8] and Martin F. Breed[1]

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Throughout this chapter I use the pronoun *'we'* to acknowledge the contributions of my coauthors, following the customary etiquette in published manuscripts.

Published in *Biological Reviews* (https://doi.org/10.1111/brv.13124).

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*Biol. Rev.* (2025), **100**, pp. 1–18.
doi: 10.1111/brv.13124


# Practical applications of soil microbiota to improve ecosystem restoration: current knowledge and future directions

Shawn D. Peddle[1,*], Riley J. Hodgson[1], Ryan J. Borrett[2], Stella Brachmann[3], Tarryn C. Davies[1], Todd E. Erickson[4,5], Craig Liddicoat[1], Miriam Muñoz-Rojas[6,7], Jake M. Robinson[1], Carl D. Watson[1], Siegfried L. Krauss[4,8] and Martin F. Breed[1]

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ABSTRACT

Soil microbiota are important components of healthy ecosystems. Greater consideration of soil microbiota in the restoration of biodiverse, functional, and resilient ecosystems is required to address the twin global crises of biodiversity decline and climate change. In this review, we discuss available and emerging practical applications of soil microbiota into (*i*) restoration planning, (*ii*) direct interventions for shaping soil biodiversity, and (*iii*) strategies for monitoring and predicting restoration trajectories. We show how better planning of restoration activities to account for soil microbiota can help improve progress towards restoration targets. We show how planning to embed soil microbiota experiments into restoration projects will permit a more rigorous assessment of the effectiveness of different restoration methods, especially when complemented by statistical modelling approaches that capitalise on existing data sets to improve causal understandings and prioritise research strategies where appropriate. In addition to recovering belowground microbiota, restoration strategies that include soil microbiota can improve the resilience of whole ecosystems. Fundamentally, restoration planning should identify appropriate reference target ecosystem attributes and – from the perspective of soil microbiota – comprehensibly consider potential physical, chemical and biological influences on recovery. We identify that inoculating ecologically appropriate soil microbiota into degraded environments can support a range of restoration interventions (e.g. targeted, broad-spectrum and cultured inoculations) with promising results. Such inoculations however are currently underutilised and knowledge gaps persist surrounding successful establishment in light of community dynamics, including priority effects and community coalescence. We show how the ecological trajectories of restoration sites can be assessed by characterising microbial diversity, composition, and functions in the soil. Ultimately, we highlight practical ways to apply the soil microbiota toolbox across the planning, intervention, and monitoring stages of ecosystem restoration and address persistent open questions at each stage. With continued collaborations between researchers and practitioners to address knowledge gaps, these approaches can improve current restoration practices and ecological outcomes.

*Key words*: ecosystem restoration, improved ecological outcomes, positive soil legacy, recovery trajectory, restoration genomics, restoration methods.

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Soil microbiota are important components of healthy ecosystems. Greater consideration of soil microbiota in the restoration of biodiverse, functional, and resilient ecosystems is required to address the twin global crises of biodiversity decline and climate change. In this review, we discuss available and emerging practical applications of soil microbiota into (*i*) restoration planning, (*ii*) direct interventions for shaping soil biodiversity, and (*iii*) strategies for monitoring and predicting restoration trajectories. We show how better planning of restoration activities to account for soil microbiota can help improve progress towards restoration targets. We show how planning to embed soil microbiota experiments into restoration projects will permit a more rigorous assessment of the effectiveness of different restoration methods, especially when complemented by statistical modelling approaches that capitalise on existing data sets to improve causal understandings and prioritise research strategies where appropriate. In addition to recovering belowground microbiota, restoration strategies that include soil microbiota can improve the resilience of whole ecosystems. Fundamentally, restoration planning should identify appropriate reference target ecosystem attributes and – from the perspective of soil microbiota – comprehensibly consider potential physical, chemical and biological influences on recovery. We identify that inoculating ecologically appropriate soil microbiota into degraded environments can support a range of restoration interventions (e.g. targeted, broad-spectrum and cultured inoculations) with promising results. Such inoculations however are currently underutilised, and knowledge gaps persist surrounding successful establishment in light of community dynamics, including priority effects and community coalescence. We show how the ecological trajectories of restoration sites can be assessed by

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# CONTENTS

# I. INTRODUCTION

Overexploitation of natural systems has led to the biodiversity crisis (Ceballos *et al.*, 2015; Dirzo & Raven, 2003) and vast areas of degraded ecosystems (Gibbs & Salmon, 2015). While conserving remnant ecosystems is a priority, there is also a need to restore degraded areas to biodiverse and functioning ecosystems (Higgs *et al.*, 2018; Perring, Erickson & Brancalion, 2018; Moreno-Mateos *et al.*, 2020). Accordingly, there is a marked increase in ecosystem restoration globally, with targets to restore more than 350 million hectares under The Bonn Challenge and the United Nations declaring 2021–2030 the Decade on Ecosystem Restoration. However, there is considerable room to improve the success of restoration projects (Crouzeilles *et al.*, 2016; Wortley, Hero & Howes, 2013).

The essential role of soil in ecosystem restoration is recognised, mainly by considering soil physical and chemical processes in ecosystem recovery (Costantini *et al.*, 2016; Muñoz-Rojas, 2018; Perring *et al.*, 2015). Over the last 15 years however, increased attention has been given to soil microbiota – the communities of bacteria, archaea, fungi, viruses and protists within soils – and their interactions in the soil system and with aboveground biota due to their essential functional roles (Harris, 2009; McKinley, 2019; Eisenhauer *et al.*, 2017a). Soil microbiota are among the most biodiverse and functionally important ecosystem components and are essential to many biogeochemical processes. For example, biological nitrogen fixation by diazotrophs, nitrogen-fixing bacteria and archaea forms the foundation of Earth's terrestrial productivity (Zhu *et al.*, 2022; Vitousek *et al.*, 2013) and cyanobacteria (carbon and nitrogen fixers) combine with fungi, bacteria, lichens, and other organisms to form biological soil crusts ('biocrusts') which can stabilise soil

587 landscapes and enhance water availability (Weber *et al.*, 2022; Yan-Gui *et al.*, 2013).
588 Furthermore, soils are home to over half of Earth's biodiversity (Anthony *et al.*, 2023)
589 and belowground microbial biomass is often comparable in scale to aboveground
590 plant or animal biomass (Fierer, 2017). Soil microbiota also interact with
591 aboveground ecosystem components and are intimately involved in plant and animal
592 health, and *vice versa*. For example, the relationship between plants and arbuscular
593 mycorrhizal fungi is one of the oldest terrestrial symbiotic interactions (Field &
594 Pressel, 2018; Tisserant *et al.*, 2013) where plants depend on fungi to gather
595 essential nutrients in exchange for carbohydrates. Consequently, we can expect
596 reciprocal shifts in above and belowground ecosystem components (Kardol &
597 Wardle, 2010; Prober *et al.*, 2015). Therefore, improving the integration of soil
598 microbiota and associated microbial ecology into ecosystem restoration will have
599 considerable benefits across restoration planning, intervention, and monitoring
600 phases (Fig. 1).

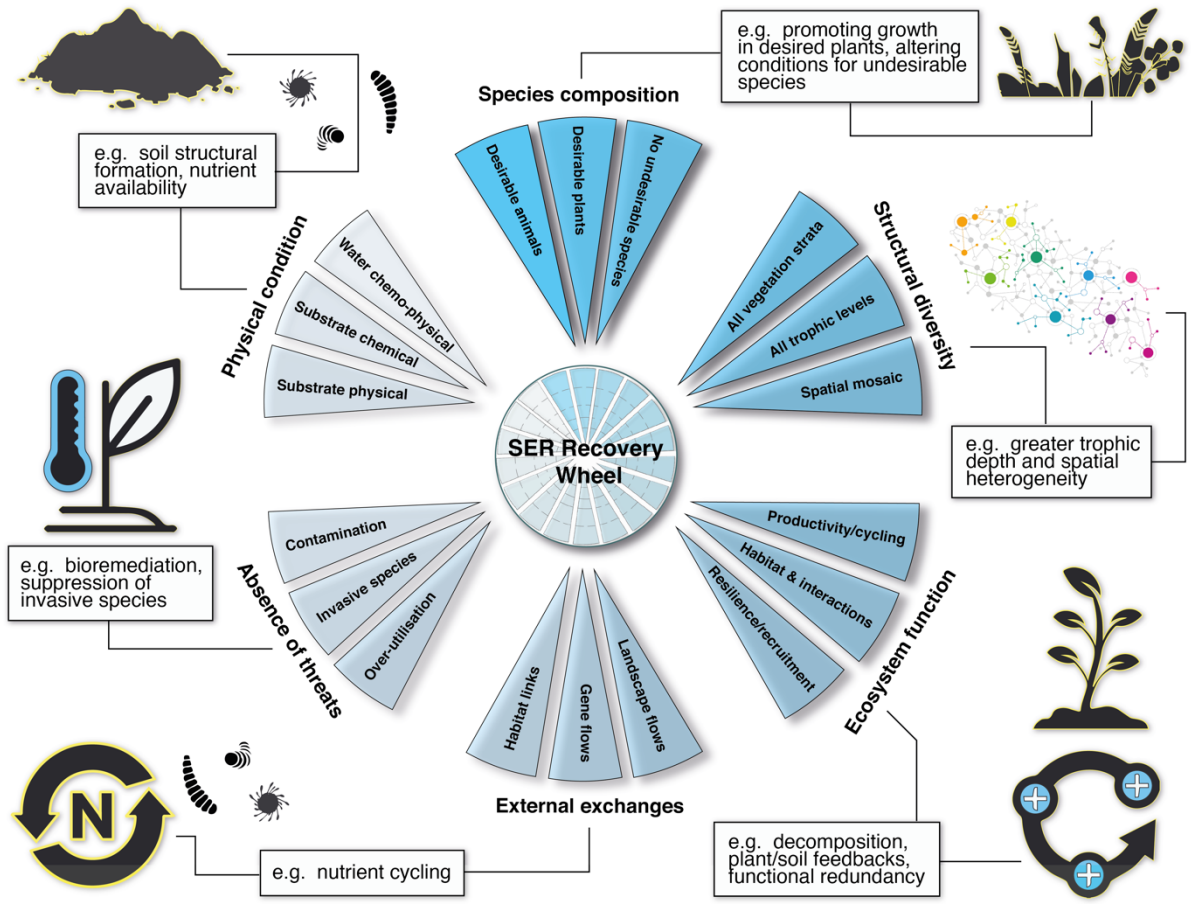


***Fig. 1.*** *The Society for Ecological Restoration (SER) recovery wheel (Gann et al., 2019) and how improved integration of soil microbiota into the planning, intervention, and monitoring phases of ecosystem restoration projects could contribute to each of the six recovery outcome themes.*

Historically, scientists faced technological challenges in quantifying and grasping the diversity and composition of soil microbiota, as traditional culture-dependent methods were only able to grow <1% of microbial taxa (Alivisatos *et al.*, 2015; Vartoukian, Palmer & Wade, 2010). However, modern sequencing technologies enable a detailed taxonomic and functional understanding of soil microbiota. For example, the now routine high-throughput amplicon sequencing of DNA extracted from soil samples can provide a detailed taxonomic view of the microbiota within a given sample (Berg *et al.*, 2020; Fierer, 2017). These amplicon datasets can then be

615 associated with spatial, land-use, environmental condition and/or restoration-
616 intervention data to answer ecological questions (Tedersoo *et al.*, 2019; Thomsen &
617 Willerslev, 2015; Breed *et al.*, 2019).
618
619 Advances in DNA-based technologies and improvements in our understanding of
620 plant–soil–ecosystem interactions are enhancing our ability to use soil microbiota in
621 restoration (Mohr *et al.*, 2022). Indeed, there are several reviews on soil microbiota
622 in a restoration context, and most have focused on theoretical aspects of including
623 soil microbiota in restoration or relevant technological advancements (Coban, De
624 Deyn & van der Ploeg, 2022; Contos *et al.*, 2021; Rawat *et al.*, 2022). Here, we
625 complement these previous reviews by focussing on the practical interface of soil
626 microbiota and ecosystem restoration and highlight key knowledge gaps that are
627 limiting effective integration of soil microbiota into restoration. We highlight where
628 and how the integration of soil microbiota has successfully occurred and identify
629 opportunities and challenges for improved integration to enhance restoration
630 outcomes.
631

## 632 II. SOIL MICROBIOTA IN RESTORATION PLANNING

633 Planning a restoration project requires setting realistic goals, making informed
634 choices of interventions, and deciding on indicators to monitor progress towards
635 stated goals (Hobbs & Norton, 1996; Collen & Nicholson, 2014; Suding *et al.*, 2015).
636 Unfortunately, despite enormous growth in the scope and scale of restoration
637 globally, many projects fail to achieve their stated goals despite the growing scientific
638 rigour of restoration practice (Crouzeilles *et al.*, 2016; Wortley *et al.*, 2013; Sun *et al.*,
639 2017). The reasons for these shortfalls are numerous and include insufficient

consideration of soil microbiota (Heneghan *et al.*, 2008; Kardol & Wardle, 2010; Farrell *et al.*, 2020). Accordingly, restoration projects should routinely consider soil microbiota early as part of their *modus operandi* – together with more traditional targets and assessments for flora and fauna. These projects will then be in a better position to determine their ecological starting place, trajectory and target – all components of best-practice restoration (Kardol & Wardle, 2010; Heneghan *et al.*, 2008; Gann *et al.*, 2019b). Here, we outline how and when restoration projects should plan to incorporate soil microbiota from the outset to maximise benefits to ecological outcomes while avoiding wasted resources. We also highlight that improving our understanding of how specific restoration interventions affect soil microbiota is needed to plan restoration effectively.

**(1) Considering soil microbiota and restoration goal setting**

Quantifying the severity of the degradation of an ecosystem is crucial in determining the level of intervention required to meet targets (Heneghan *et al.*, 2008; Chazdon, 2008). For example, when a restoration site is depleted of mycorrhizal fungi required by a target plant species (e.g. mixotrophic orchid species are entirely dependent on orchid mycorrhiza for germination), there is little sense in investing resources to establish the plant without simultaneously addressing the lack of symbiotic fungi (Koziol, Crews & Bever, 2020). Furthermore, invasive plant species in a degraded landscape can modify soil microbiota to the point that the soil environment is in an alternate state of dynamic equilibrium (Suding, Gross & Houseman, 2004; Gornish *et al.*, 2020). Here, removing the invasive plants and revegetating the landscape relies on the soil microbiota to move towards a state that is more supportive of the recovering native plant community which is by no means guaranteed (Harris, 2009).

A revegetation-only approach may not overcome persistent soil legacies (i.e., altered nutrient levels from fertiliser use, altered soil structure from compaction, invasive species, undesirable biological communities) and risks perpetual states of ecological invasion (Anthony *et al.*, 2019; Bell, Siciliano & Lamb, 2020). As a result, specific interventions that address invasive plants and altered soil microbiota (see Section III) need to be part of the restoration planning phase. Moreover, major disturbance to soil physical and chemical conditions (e.g. from mining, erosion, compaction, excess nutrients) will alter the foundational habitat for soil microbiota, so addressing limiting abiotic factors also represents a key priority in restoration planning (Robinson *et al.*, 2024b). There is immense value in setting early goals to understand soil microbial ecology at the initial stages of a restoration project. This goal-setting process will help the restoration practitioner to quantify and pre-empt biotic and abiotic constraints or opportunities (e.g. a lack of mycorrhizal fungi, plant-associated pathogens for structuring plant communities, altered soil physical or chemical properties).

If barriers to recovery are not identified as part of the planning stage, ecosystem recovery will likely be inhibited (Hobbs & Norton, 2004). Practitioners should address these constraints in a restoration project by, for example, using knowledge of plant–soil feedbacks in the planning phase. Restoration projects could promote negative feedbacks between plant and soil communities by, for example, inoculating sites with late-succession soil microbiota that encourages vegetation diversity in the early recovery phase (Carbajo *et al.*, 2011; Kardol, Martijn Bezemer & Van Der Putten, 2006). This can lead to mycorrhizal fungi outpacing bacterial pathogens, potentially promoting community evenness in late-succession plants (Fierer, 2017; Kardol &

690 Wardle, 2010). Integrating soil microbial ecology knowledge into predictive ecological
691 frameworks (e.g. modelling different environmental change scenarios, including
692 microbiota assembly and functional dynamics) could further allow targeted site-
693 specific restoration plans (Eviner & Hawkes, 2008).
694
695 Reference site selection and assessments are central elements of planning and
696 defining goals in a restoration project (Gann *et al.*, 2019b). Soil physical and
697 chemical conditions, together with plant diversity and other factors in reference sites,
698 shape microbiota development (Fierer, 2017). While reference site soil microbiota
699 are increasingly used in restoration monitoring (see Section IV), they are not
700 routinely assessed during the planning phase. Gaining information on the
701 composition, and even functional characteristics, of microbiota in both degraded and
702 target reference sites will position projects better to tailor their interventions to
703 address varied levels of degradation in the whole ecosystem. Soil microbiota are
704 highly heterogeneous across even small (<1 $cm^2$) spatial scales (Fierer, 2017) and
705 therefore reference site selection and sampling design are crucial to capture
706 variation adequately (van der Heyde, Bunce & Nevill, 2022; Liddicoat *et al.*, 2022).
707 This high level of spatial variation can impact assessments of community
708 composition and function, and distort interpretations of the reference community
709 (Peddle *et al.*, 2022).
710
711 The numbers and locations of reference site samples should account for vegetation
712 and soil heterogeneity to provide the best possible picture of microbiota targets
713 (Peddle *et al.*, 2022; van der Heyde *et al.*, 2022). One option is to implement a
714 stratified random sampling scheme. This approach involves dividing the study area

into distinct strata based on relevant factors influencing biodiversity distribution, such as vegetation and soil types or topographical features. Within each stratum, random sampling points are selected to ensure representative coverage of the area while minimising bias and distortions caused by heterogeneity. Additionally, employing systematic sampling techniques, such as grid or transect sampling or pooling samples to account for landscape heterogeneity (Bissett *et al.*, 2016) can further enhance spatial representativeness and accuracy of biodiversity assessments.

A key open question in integrating soil microbiota into restoration is: what do 'good' soil microbial communities look like in terms of species composition and/or functionality? The composition of soil microbiota will vary greatly in different contexts and environments with no single 'ideal' microbial community (Fierer, Wood & de Mesquita, 2021). Generally speaking, the microbial community composition most suited for any given restoration site should be informed by suitable reference sites. However, understanding the specific elements of microbial communities and drivers of microbial diversity, composition and function that can be generalised across environments will improve how and where we integrate microbiota into restoration (Liddicoat *et al.*, 2024). In some cases, desirable microbiota characteristics might be informed by higher-level functional outcomes (e.g. establishment of sensitive plants, nutrient cycling, disease suppression). Various microbial taxa have seen increased research focus on their uses for restoring particular ecosystem processes or connections. For example, plant growth-promoting rhizobacteria have potential for their ability to improve plant growth (Radhapriya, Ramachandran & Palani, 2018; Solans, Pelliza & Tadey, 2022) and enhance germination (Domínguez-Castillo *et al.*, 2021), and arbuscular mycorrhizal fungi can promote recovery of native vegetation

*via* mechanisms that enhance phosphorous uptake in plants (Koziol *et al.*, 2018) and improve soil physicochemical properties (Willis, Rodrigues & Harris, 2013).

While there are 'good' members of microbial communities, there are also pathogens that can be harmful to microbial communities and other ecosystem components (e.g. *Phytophthora cinnamomi* is a soil-borne plant pathogen) (Mansfield *et al.*, 2024) and restoration plans need carefully to consider the risks of inadvertently spreading harmful pathogens. Importantly however, plant–pathogen interactions may be beneficial in restoration as they also play a significant role in shaping plant diversity and community dynamics. Pathogens can influence plant diversity through various mechanisms, including selection pressure on host species and facilitation of competitive interactions (Bever, Mangan & Alexander, 2015), which may impact plant and soil community stability.

**(2) When to prioritise investment in soil microbiota**

Another key question that needs to be addressed to ensure restoration is as efficient and effective as practically possible is: when will the inclusion of soil microbial data improve restoration success? While the explicit consideration of soil microbiota in restoration could arguably provide benefits to all projects, it does come with additional costs (e.g. soil sampling, DNA extraction and sequencing, complex bioinformatics) and potential risks (e.g. introduction of harmful pathogens, public or policymaker scepticism from undesirable outcomes) that need to be considered to maximise positive restoration outcomes and avoid wasted resources. Soil ecosystems are complex and highly variable both within and across sites which means a one-size-fits-all recommendation is problematic. Furthermore, soil

765 microbiota are unlikely to be the only factor hindering restoration outcomes.
766 Restoration projects should therefore include risk assessments and cost–benefit
767 analyses on a case-by-case basis to determine if, and to what extent, soil microbiota
768 should be included. The inclusion of soil microbiota in any given restoration project
769 and any determination on the likelihood of that inclusion translating into cost-effective
770 improved restoration outcomes will be largely dependent on the project's goals and
771 level of degradation or disturbance of soil physical, chemical, and biological
772 properties.
773
774 Decisions on including soil microbiota in restoration plans and interventions should
775 be informed largely by the impact that degrading processes have had on soils and
776 the level of investment that is available. Even short-term disturbances to vegetation
777 communities with minimal disturbance to soils can cause shifts in soil microbial
778 diversity and composition (Navarrete *et al.*, 2015; Qu *et al.*, 2024). However, if soil
779 physical and chemical properties remain similar to an undisturbed state, a focus on
780 restoring vegetation communities alone may be sufficient to see the recovery of soil
781 microbiota. On the contrary, if degrading processes have substantially modified soil
782 physical or chemical properties, then soil biological properties will most likely be
783 impacted as well. For example, restoration sites that were previously used for
784 agriculture with extensive fertiliser applications can have long-lasting nutrient
785 legacies that persist for decades to millennia (Turley *et al.*, 2020; Parkhurst, Standish
786 & Prober, 2022b).
787
788 These persistent land-use legacies can then act as an abiotic barrier and impede the
789 recovery of soil microbiota and present situations where restoration should plan

interventions that specifically seek to overcome these abiotic constraints (Peddle *et al.*, 2024a). Additionally, alterations in soil pH, moisture, and structure resulting from degradation can also influence microbial community composition and activity, further emphasising the relevance of soil physicochemical assessments in guiding the inclusion of soil microbiota in restoration initiatives. Physical and chemical conditions are generally easier to observe and test than soil microbiota and should be considered to provide as near-optimal conditions as possible with reference sites as a guide. This sets the foundation for development of biological communities (Robinson *et al.*, 2024b). Restoration planning can, of course, consider 'in-principle' influences on (and *via*) microbiota, however, practitioners will be blind to actual effects and recovery if relevant attributes of microbiota remain uncharacterised. By integrating assessments of soil physical, chemical, and biological properties, restoration practitioners can tailor decisions on the inclusion of microbiota-based interventions (see Section III) to the specific needs of degraded ecosystems, facilitating more effective restoration outcomes.

**(3) Improving conclusions on causation in soil microbiota restoration**

To determine better the level of effort required to affect recovery of soil microbiota it is important to improve our understanding of how soil microbiota responds following traditional restoration interventions such as revegetation. If revegetation alone largely leads to recovery of soil microbiota, then costly assessments and interventions focussed on microbiota are probably not needed. However, attributing soil microbial recovery solely to revegetation without properly ascertaining causation will lead to soil microbiota being overlooked and risks missing opportunities either to address this crucial ecosystem component directly (Lem *et al.*, 2022) or to utilise soil

815 microbiota more as drivers of change as opposed to solely passengers (Harris,
816 2009). Observational studies of soil microbiota following revegetation often indicate
817 that soil microbiota in restoration sites resemble reference sites more closely with
818 increasing time since restoration (Barber *et al.*, 2017; Gellie *et al.*, 2017; Klopf *et al.*,
819 2017; Ngugi *et al.*, 2018; Parsons *et al.*, 2020; Sun *et al.*, 2017; Yan *et al.*, 2019;
820 Banning *et al.*, 2011). These studies are often used to infer that the restoration
821 intervention (e.g. native plant revegetation) is *causing* the restoration of soil
822 microbiota but further rigour is needed to improve our knowledge of causal
823 mechanisms affecting the recovery of soil microbiota.
824
825 Observational chronosequence-based studies often suffer from unmeasured or
826 unaccounted factors that can confound results and cloud conclusions. Other soil
827 characteristics (both biotic and abiotic), climate, aboveground biological influences
828 (e.g. vegetation, land-management history), topographic relief, parent geological
829 materials, age of development, and spatial location (e.g. proximity to external
830 influences) will influence soil microbiota composition (Delgado-Baquerizo *et al.*,
831 2020; McBratney, Santos & Minasny, 2003; Pino *et al.*, 2019) and may vary
832 independently of a restoration intervention. Furthermore, restoration methods may
833 change over time; for example, an unpredictable supply of seed resources may
834 cause temporal variation in revegetation (Broadhurst *et al.*, 2016; Ladouceur *et al.*,
835 2018), or there may be inter-seasonal changes in climate, or changes in revegetation
836 practices or planting crew. These time-dependent changes to restoration practice
837 can introduce uncontrolled variation across the chronosequence and must be better
838 considered during chronosequence studies. In many situations, collecting sufficient
839 covariate data to explain fully (or develop models to account for) soil microbiota

spatial autocorrelations is impractical. Therefore, ensuring that these unmeasured or unaccounted influences do not compromise experimental designs and sampling plans by having appropriately designed studies is necessary.

Despite their limitations, chronosequence designs are useful for inferring ecological responses to restoration interventions through time without long-term sampling or controlled experiments (Walker *et al.*, 2010). However, explicitly planning to embed good quality experiments – such as those with adequate replication, controls and randomisation – into restoration projects will help to alleviate issues with spatial autocorrelation (van der Heyde *et al.*, 2022) or pseudo-replication (i.e., treatment $N$ = groups of 1) and assist in minimising the effects of confounding factors (e.g. changes in restoration planting methods, seed supply, climate variation, spatial location). However, it should be noted that truly longitudinal and/or manipulative studies are needed to produce high-quality evidence and conclusive support on causation (Lem *et al.*, 2022) (see Section II.5).

**(4) Embedding soil microbiota experiments**

By planning to embed well-designed experiments into restoration projects, practitioners and researchers could form partnerships to address many of the limitations of chronosequence (i.e., space-for-time) designs (Broadhurst *et al.*, 2023). Embedded experiments could nest replicated soil microbiota interventions (e.g. different soil inoculation methods or revegetation techniques) within reference and restoration sites or include spatially independent and replicated restoration interventions across a project (Fig. 2). Such an approach will improve the evidence

base of the effect of specific restoration interventions on the recovery of soil microbiota and their associated functions.

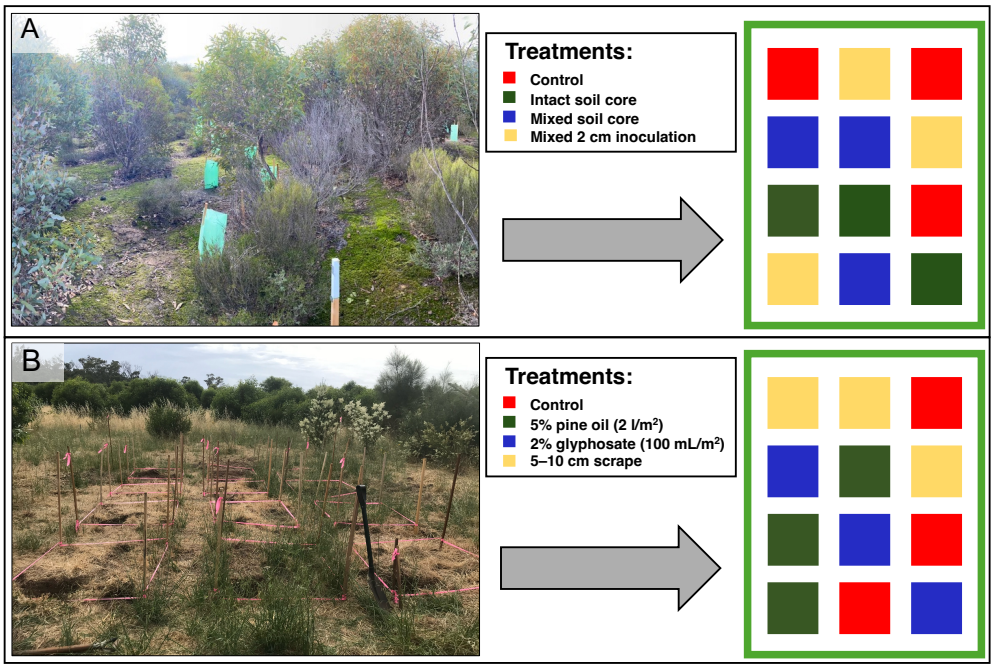


***Fig. 2.*** *Embedding soil microbiota experiments into restoration sites. (A) A soil microbiota translocation field experiment embedded into an ongoing restoration project in Western Australia [photograph credit: Shawn Peddle]. (B) Embedded soil microbiota experiments in a restoration site in the Mt Lofty Ranges, South Australia [photograph credit: Tarryn Davies]. Designing and embedding experiments into restoration projects will allow for improved causal conclusions in testing microbiota-focussed hypotheses.*

Adequately replicated, randomised, controlled and comparable restoration sites are not often routinely present in restoration projects unless planned for from the outset. This lack of core scientific design principles in systems that are often used in observational studies limits conclusions that can be drawn from such research. For this reason, restoration projects could improve our evidence base by collaborating with researchers and embedding microbiota-focussed experiments into restoration

projects. Well-designed longitudinal studies that repeatedly sample the same restoration sites through time will provide more robust evidence on cause–effect relationships than cross-sectional chronosequence studies alone (Christie *et al.*, 2019; Lem *et al.*, 2022). Embedding experiments would also help close critical restoration knowledge gaps, such as knowing when a focus on soil microbiota will substantially improve restoration success. However, by their very nature, longitudinal studies require years of research and given the urgency required to address the biodiversity crisis, statistical modelling methods (e.g. structural–causal modelling, see Section II.5) will be useful to help understand the key knowledge gaps that need to be addressed with on-site long-term experiments and what can be solved with observational study designs alone.

**(5) Modelling approaches to ascertain causation**

Where particular microbiota-oriented outcomes are desired, but restoration activity cannot wait for definitive experimentally derived knowledge on cause–effect relationships, certain modelling approaches may help to distil information from relevant existing microbiota-restoration datasets. Techniques such as structural–causal modelling, structural equation modelling and path analysis can be applied to test hypotheses using observational cross-sectional data (Arif & MacNeil, 2023; Grace & Irvine, 2020). These approaches involve specifying a theoretical model that reflects likely causal relationships among variables of interest, including both observed and latent (i.e., unmeasured) variables. Then, the hypotheses are tested by specifying directional paths that represent the assumed causal relationship between variables in the model. Using the observed data, parameters (i.e., coefficients) of the specified model are estimated. These estimates assess the

907 strength of the hypothesised causal/directional relationships and goodness of fit
908 metrics indicate how well the model aligns with the data, even in the absence of
909 experimental evidence (Eisenhauer *et al.*, 2015), facilitating informed decision
910 making in restoration planning.
911
912 As a specific example, we could consider a scenario where a restoration intervention
913 aims to enhance soil fertility and plant growth by introducing specific microbial
914 species or communities. By using structural causal modelling, researchers can
915 construct a theoretical model that includes variables related to soil microbiota
916 composition and function, soil fertility, and plant performance. They can hypothesise
917 directional paths between these variables representing the assumed causal
918 relationships. Through analysis of observational cross-sectional data from similar
919 restoration projects and controlling for covariates, researchers can estimate the
920 parameters of the model and assess the strength and direction of the hypothesised
921 causal relationships. For instance, it might be that certain microbial taxa or functional
922 genes are strongly associated with increased soil fertility, which in turn positively
923 impacts plant growth.
924
925 While structural–causal modelling with cross-sectional data is powerful, it has
926 limitations. It cannot establish causality as definitively as controlled or longitudinal
927 experiments, and causality may be more challenging to infer in the presence of
928 unobserved confounders especially in systems such as soil with thousands of
929 distinct taxa and many functional groups (Eisenhauer *et al.*, 2022). However, it
930 allows researchers to specify, estimate and evaluate complex causal models
931 providing insights into causal relationships without the need for experimental or

longitudinal designs. Furthermore, the strength of causal claims should always be considered in the context of the study's design and the potential presence of unobserved confounding variables.

# III. RESTORATION INTERVENTIONS THAT DIRECTLY TARGET SOIL MICROBIOTA

It is possible to manipulate soil microbiota to assist in the recovery of degraded ecosystems by reinforcing beneficial interactions between plant species and soil microbiota lost through degradation (Aghili *et al.*, 2014; van der Putten *et al.*, 2016; Albornoz *et al.*, 2022). Soil microbiota-focussed interventions can improve plant species prospects by improving plant growth, and depending on the ecological system, diverse soil microbiota have also been shown to mediate vegetation community diversity and improve ecosystem productivity (Naeem *et al.*, 1994; Yang *et al.*, 2021). Informed by mechanisms of ecosystem recovery and species-specific responses, soil microbiota interventions can advance restoration objectives and restore the diminished capacity of impacted ecosystems to recover naturally. In this section, we review more established (e.g. soil inoculations) and less well-established (e.g. specific microbial cultures, seed enhancements) ways to manipulate soil microbiota to improve restoration outcomes. We note that obvious abiotic barriers to the development of site-specific favourable soil microbial communities (e.g. soil substrate problems, excess nutrients, low pH, high salinity) should be identified and addressed before attempting direct manipulation of soil microbiota.

**(1) Whole soil translocations and microbial inoculations**

Translocating whole soil communities – whether in the form of intact turfs or homogenised bulk soil – is one way of inoculating soil microbiota into degraded ecosystems to shift the microbial community towards one that is more representative of a target ecosystem. This essentially involves collecting soil from a reference ecosystem and translocating it directly into a restoration site (Koziol *et al.*, 2018; Wubs *et al.*, 2016b; Carbajo *et al.*, 2011). Inoculating degraded sites with reference ecosystem soil and associated biota has been shown to improve the growth and establishment of desirable native plants and exclude weeds in both greenhouse and field conditions (Koziol *et al.*, 2018; Wubs *et al.*, 2016b; Fahey & Flory, 2022). For example, Wubs *et al.* (2019a) showed that soil inoculations can have ecosystem legacy effects that steer successional changes and can last for at least two decades. Importantly, however, Gerrits *et al.* (2023) highlight how the directionality of this legacy effect depends on the suitability or fit of translocated soil to the recipient site, with mismatches steering communities in the wrong direction. Similar interventions can also shift the direction of the development of vegetation communities (Wubs *et al.*, 2016b) and improve prospects for native vegetation success (Wubs *et al.*, 2019b). However, while research has shown a benefit for the restoration of vegetation, few studies have focussed on the efficacy of soil translocations to shift whole microbial communities themselves.

Substantial knowledge gaps remain on the effectiveness of soil translocations, including: what methods are best to use (e.g. bulk soil, intact turfs, volumes required), to what extent do soil physical and chemical properties in recipient sites impact establishment, how priority effects impact on microbial community recovery (i.e., establishment may be dependent on the order of arrival of specific taxa), and,

how does the coalescence of distinctly different soil communities impact successful establishment? As such, further research on whole soil translocations and inoculations should focus on addressing these knowledge gaps via embedded experiments to understand better how soil volume, translocation method, and community coalescence dynamics affect microbial community assembly across varied ecosystems and soil types. Addressing these knowledge gaps will then enable the research community to develop decision-support frameworks to help determine when whole-soil translocations will provide restoration benefits that are commensurate with cost.

Another critical open question relating to soil translocation is: how can we minimise the impacts soil translocations have on donor ecosystems? While soil translocations may be effective, soil collection can impact remnant habitats and consideration is needed to limit impacts to remnant sites while providing a benefit to degraded sites. Solutions are needed to scale up soil translocations outside situations where soil can be harvested because existing remnant habitat is already being cleared. As such, decisions on interventions impacting remnant habitat will need to weigh factors such as the contribution of remnant habitat to support the integrity and viability of restoration or conservation efforts (Tulloch *et al.*, 2016; Wintle *et al.*, 2019), or if a degree of destructive harvesting of soil resources from remnant sites can provide restoration benefits that outweigh impacts to remnant habitat. To address the need for reliable seed sourcing in restoration or revegetation, seed-production areas are being established instead of relying on sourcing seeds from remnant habitats (i.e., target plants are grown *ex-situ* ‘en masse’ to produce seed stock) (Zinnen *et al.*, 2021). This concept could potentially be applied to soil microbiota with soil

microbiota production areas, although various open questions (i.e., how do we cultivate whole target microbial communities, can we subset communities to focus on particular taxa, and what is the 'ideal' composition of these communities) need to be addressed before soil microbiota production areas can be effectively implemented at scale.

Despite these knowledge gaps, whole-soil translocations are increasingly used in large-scale restoration projects where topsoil is salvaged as part of the initial disturbance (e.g. surface strip mining) and then reinstated during restoration (Tibbett, 2010; Schmid *et al.*, 2020; Liddicoat *et al.*, 2022). The objective of topsoil transfer is to preserve the soil-stored seedbank rather than the soil microbiota *per se*. Still, benefits from the reservoir of microbiota contained in these topsoils present an opportunity to improve restoration outcomes. Limiting the amount of time for which soils are stockpiled before translocation is crucial as stockpiling can disrupt biological integrity and impact microbial diversity and composition (Hernandez *et al.*, 2024; Valliere *et al.*, 2022). In best-practice cases, the direct return of harvested topsoil to nearby restoration sites will limit the physical and biological degradation of soil from long-term stockpiling (Rokich *et al.*, 2000; Peddle *et al.*, 2022). However, the impact of the collection and homogenisation of vertical soil profiles during the transfer process on soil microbiota is likely detrimental but still poorly understood.

An emerging approach that avoids broadacre spreading of whole soil is the targeted use of microbiota from local soils *via* extruded pellets or coatings as a vessel for seed delivery (Gornish, Arnold & Fehmi, 2019; Madsen *et al.*, 2016) (Fig. 3). This method is designed to improve the precision of seed delivery in large-scale

restoration efforts that simultaneously provide beneficial soil microbiota and the target seeds. Such an approach can reduce the demand for soil by 100-fold (Stock *et al.*, 2020). However, similar questions to those identified earlier in relation to whole-soil inoculations are still unresolved and applicable here. For example: how do the mechanical and chemical disturbances of creating the pellets affect microbial community composition, and how well can targeted microbial communities in extruded pellets be established within donor soils with vastly different microbial communities or physicochemical properties?

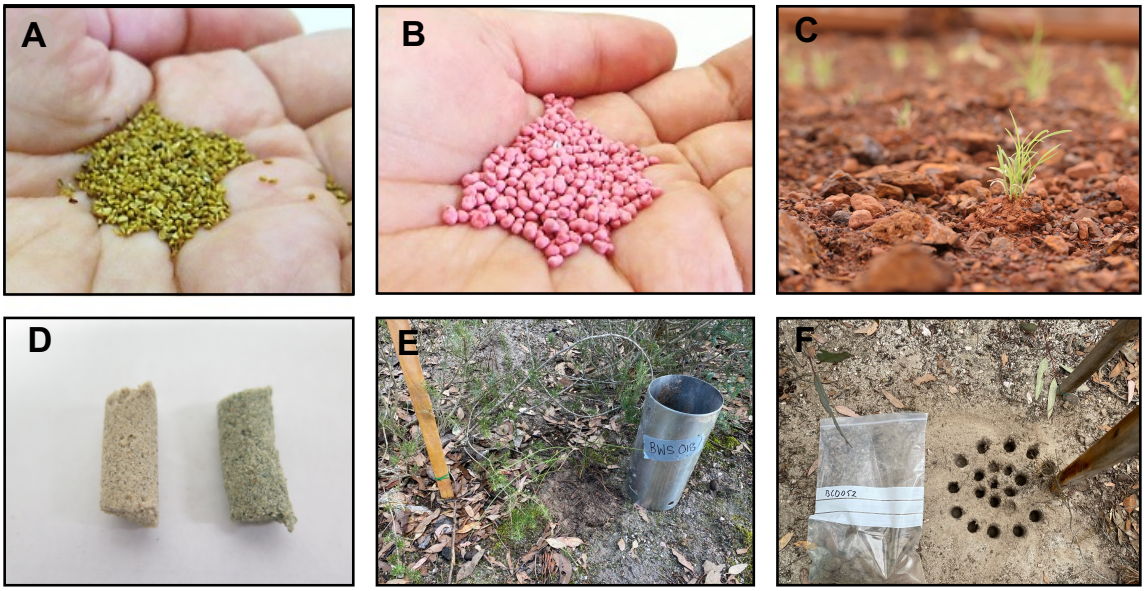


***Fig. 3.*** *Manipulating soil microbiota as part of restoration interventions. Eucalyptus seeds (A) before and (B) after coating with mycorrhizae inoculants [photograph credit: Todd Erickson]. (C) Seedlings germinated from extruded seed pellets containing live soil [photograph credit: Todd Erickson]. (D) Control (sand + bentonite, left) and cyanobacteria encapsulated pellets (right) [photograph credit: Miriam Muñoz-Rojas]. (E, F) Whole soil translocation experiment with (E) translocated intact soil core and (F), resampling one year after translocation to assess establishment and dispersal of soil microbiota [photograph credit: Shawn Peddle].*

**(2) Seed enhancements that contain microbial additives**

Seed enhancements that add specific microbial inoculants in a restoration context can improve the germination and growth of desirable plant species (Chua *et al.*, 2019; Dadzie *et al.*, 2024; O'Callaghan, 2016). Seed coating involves the precise application of binders and mineral powders to seeds to create a thin artificial layer of material capable of altering the physical shape of seeds and/or carrying beneficial products such as microbiota (Fig. 3 A–C) (Brown *et al.*, 2021; Erickson *et al.*, 2021). Similarly, extruded pellets made *via* extrusion or moulding technologies can make larger seed-soil matrices while offering the same microbial inoculation opportunities. For instance, microbiota can be added to the seed coat and/or into extruded pellet ingredients either dry within the powder or wet via the binder (Alfonzetti *et al.*, 2023; Dadzie *et al.*, 2024; Munro *et al.*, 2024). Alternatively, seed priming involves immersing seeds in water-based (i.e., hydro-priming) or osmotically controlled (i.e., osmo-priming) solutions or soil matrix (i.e., matrix-priming) to commence the process of germination under controlled conditions, followed by a re-drying step (Brown et al., 2021; Madsen et al., 2018). Once sown, primed seeds show a much higher and more rapid germination potential, recruitment synchronicity and seedling vigour. Priming with additives like microbial suspensions can facilitate the uptake of beneficial microbiota directly onto and potentially into the seeds (O'Callaghan, 2016; Muñoz-Rojas, 2018). Consequently, the targeted microbiota will be established directly in the soil near the germinating seed or within the seedling tissue itself (O'Callaghan, 2016; Chua *et al.*, 2019).

There have been successful implementations of naturally-obtained microbiota that benefit key restoration plant species by seed enhancements (including extruded

seed pelleting and/or hydro-priming with microbial additives) (Muñoz-Rojas *et al.*, 2018; Dadzie *et al.*, 2022). The improved accessibility and effectiveness of these techniques represent a valuable opportunity for restoration. Bioencapsulation – which involves encasing seeds in protective polymers (natural and/or synthetic) that sustain microbial inoculants – can also be used to enhance the survival of microbiota during storage and introduction into field soils (Brown *et al.*, 2021; Schoebitz, López & Roldán, 2013). These methods may be more suitable for large-scale field settings through the gradual and prolonged release of target microbiota. These extruded pellet or seed coatings can be applied widely *via* targeted dispersal across restoration sites to improve seed vitality and establish beneficial and targeted microbes, such as cyanobacteria for forming soil biocrusts (Román *et al.*, 2020). Additionally, using materials such as clay, carbohydrates and/or hydrogels to encase seeds and engineer seed microenvironments represents a recent technological transfer into restoration from the agricultural and pharmaceutical sectors, and shows promise for improving restoration success *via* the incorporation of microbiota (Zvinavashe *et al.*, 2019). Still, limitations preventing the implementation of these approaches include the challenge of identifying appropriate inoculants across different ecological communities, accumulating sufficient biomass, and testing these applications under field conditions. Field testing must be improved before this technology can provide a reliable and scalable tool for restoration practitioners (Ayuso *et al.*, 2017; Román *et al.*, 2018).

**(3) Microbial cultures and suspensions**

Microbial cultures and suspensions are additional alternative and promising lab-based approaches to whole-soil inoculations and seed enhancement technologies.

They rely on less-destructive sourcing of soil microbiota requiring smaller quantities of soil and, therefore, have a reduced impact on reference ecosystems compared with whole-soil translocations (Dadzie *et al.*, 2022; Muñoz-Rojas *et al.*, 2018; Román *et al.*, 2020). Microbial cultures can be isolated into a liquid medium *via* suspensions. These suspensions and cultures offer versatility: they could introduce specific microbiota into liquids or pastes and inoculate plant tissues like roots, seeds, or soils directly (Olle & Williams, 2013; Vassilev *et al.*, 2020). Isolation and culturing of soil microbiota within the context of native plants has resulted in increased recruitment and growth of a variety of plant taxa [e.g. mangroves, legumes, cacti; (Bacilio, Hernandez & Bashan, 2006; Bashan *et al.*, 2012; Radhapriya *et al.*, 2018)]. Expected applications of strain-culturing could further improve the cultivation and recovery of rare and endangered plants (Dutta, Na & Lee, 2021) and are commonplace within orchid fungal symbiosis research (Hossain, 2022). However, the successful establishment of inoculants is highly dependent on priority effects, niche/resource availability, and community cohesiveness (Debray *et al.*, 2021; Diaz-Colunga *et al.*, 2022). Limitations to these approaches include their poor shelf-life and the inadvertent loss of desirable microbiota (Ramakrishna, Yadav & Li, 2019). Moreover, many mass-produced inocula are likely unsuitable for natural environments (Koziol *et al.*, 2018; Jiang *et al.*, 2023; Kaminsky *et al.*, 2019). These techniques, therefore, require additional investment to improve their reliability, affordability, and broad-scale application if they are going to be of general use in restoration. In addition, the cost and technical challenges of matching isolated cultures and suspensions to a specific restoration context is a hurdle to overcome.

**(4) Targeting specific microbiota**

Specific microbial taxa can be lacking in an ecosystem, disproportionally impacting plant fitness (Thrall *et al.*, 2001). For example, obligate symbionts (e.g. rhizobia) often fail to persist in degraded soils since their survival relies on the presence and persistence of their host plant species (Thrall *et al.*, 2001; Berruti *et al.*, 2016) or other microbes within a whole community. For bulk soil inoculations, microbiota specificity is low as this approach relies on a whole-of-community transfer. Therefore, varying degrees of specificity are relied upon for microbial cultures and suspensions when targeted for use as an additive in direct soil applications or *via* priming, coating and extruded pelleting approaches. Because of this variable specificity, the required level of microbe–host matching is an important factor to consider when developing soil microbiota interventions (e.g. does an inoculum need to land precisely within the root zone of a target plant to succeed?). Furthermore, reliance on expensive and highly technical approaches could be a liability for restoration practice where patents and corporate control of technology could limit affordable uptake and equitable use of tools needed to improve restoration outcomes (Osborne *et al.*, 2021).

A targeted consortium of microbes (e.g. multiple taxa of cyanobacteria) may be preferable over individual strains (Chua *et al.*, 2019; Dadzie *et al.*, 2022), especially since a diverse community should result in more resilient microbiota (Chua *et al.*, 2019; Rodriguez & Durán, 2020; Berendsen *et al.*, 2018). Culturing diverse microbial consortia can be challenging however, as varying capture and growth rates across taxa are likely (Kaminsky *et al.*, 2019). A further roadblock is selecting the appropriate techniques to capture, extract and transfer the targeted microbiota or

strains. This will be particularly challenging for obligate symbionts, which can be particularly hard to isolate and culture (Berruti *et al.*, 2016).

The use of plant hosts has been proposed as a way to culture a targeted microbiota. Trap cultures in soil, for example, involve collecting soil samples containing target microbial communities – such as arbuscular mycorrhizal fungi – from whole soil in a reference ecosystem, which is then propagated with host plants *ex-situ* for later inoculation (Koziol *et al.*, 2018). Techniques like this could be scaled up in soil microbiota production areas which could reduce impacts on remnant ecosystems comparatively to the direct transfer of topsoil. However, these soil culturing systems require substantial time and technical investments to establish them. Once operational, communities may shift away from their 'wild type' or desired community. Evidence suggests that these communities can change to undesirable states over time, due to the build-up of soil pathogens (Bauer, Mack & Bever, 2015) or reduced diversity within the microbial communities, which could harm host plants (Trejo-Aguilar *et al.*, 2013) undermining the effectiveness of microbial products. Alternatively, harnessing the positive soil legacies of plants and host-mediated microbiome engineering have been proposed as methods of selecting for specific functional outcomes in microbial communities by subjecting plants to specific selective pressures (e.g. inducing drought tolerance in a host-plant's microbiota *via* instigating water stress) (Mueller & Sachs, 2015; Pineda, Kaplan & Bezemer, 2017; Gopal & Gupta, 2016). However, our ability to introduce targeted microbiota or a select microbial strain depends on our capacity to identify specific taxa of interest and extract, propagate, and successfully re-introduce them effectively and in a replicable way.

**(5) Promoting positive soil legacies**

Utilising the positive soil legacies of plants could improve the fitness of plant species used in revegetation (Chua *et al.*, 2019; Koziol *et al.*, 2018). Positive soil legacies involve the recruitment and fostering of favourable microbiota in the soil through a preparatory generation of plant growth, thereby priming the soil microbiota to provide a plant fitness advantage for the next generation (Pineda *et al.*, 2017; Gopal & Gupta, 2016).

The potential for creating positive soil legacies through priming the soil with specific plants has been demonstrated with the wildflower *Senecio jacobaea* (Pineda *et al.*, 2017). When exposed to insect pests, this plant generated a feedback mechanism where sugars and organic acids exuded from its roots maintained a distinct soil fungal community that affected the regulation of amino acids in the host plant's phloem sap, providing the plant with reduced herbivore populations (Kos *et al.*, 2015). Also, Buchenau, van Kleunen & Wilschut (2022) observed, for some European grasses, improved growth in the second generation of plants grown in drought-exposed and nutrient-limited soils, due to a positive legacy of the soil microbiota. The next step for utilising positive soil legacies better is to improve understanding of the generality of this effect as it is not present for all plant species (Kaisermann *et al.*, 2017).

Understanding microbial-mediated stress responses in plants and how plant–microbial interactions can be applied to improve plant stress tolerance presents promising restoration opportunities (Larson, Venette & Larson, 2022; Petipas, Geber

& Lau, 2021; Valliere *et al.*, 2020). The transfer of soil microbiota from non-local soils or across environmental gradients (e.g. temperature, aridity, nutrient) into revegetation sites could instil stress-ameliorating interactions between plants and the relocated microbiota. This could build resilience to developing stress and disturbance expected under climate change or site-specific legacies of previous land use – provided we improve our understanding of patterns of host-plant-specific *versus* general adapted microbial functions (Petipas *et al.*, 2021).

# IV. MONITORING SOIL MICROBIOTA FOR RESTORATION

Accurate and efficient monitoring of biotic and abiotic responses to restoration is required to ensure that recovery is progressing as desired (Ruiz‐Jaen & Mitchell Aide, 2005; Collen & Nicholson, 2014; Muñoz-Rojas, 2018) and facilitates both the evaluation of a project against its stated goals and amendments to a project should evaluation uncover a lack of suitable progress (Gann *et al.*, 2019b; Liddicoat *et al.*, 2022). While the historical inclusion of soil microbiota in restoration goals has been rare, they are now increasingly used to assess ecosystem recovery as advances in sequencing technology improve our understanding of microbial ecology (Liddicoat *et al.*, 2022; Mohr *et al.*, 2022; van der Heyde *et al.*, 2022). Most studies have used a chronosequence design (see Section II.3) for common issues with this design) and have characterised soil microbiota using a variety of methods (Table 1). Different microbiota assessment methods have their pros and cons, which we briefly outline below with key study design considerations.

*Table 1. Key tools used to characterise microbial communities, functions and/or biomass in soil.*

| Technique | DNA based | Complexity | Data analysis | Biomass data | Taxonomic data | Functional data |
|---|---|---|---|---|---|---|
| Phospholipid fatty acid analysis (PLFA) | No | Moderate | Easy | Yes | Poor | Poor |
| Amplicon sequencing | Yes | Low | Moderate | No | Detailed | Poor to moderate |
| Shotgun metagenomics | Yes | High | Hard | No | Detailed | Detailed (potential) |
| Metatranscriptomics | Yes | Very high | Hard | No | Moderate | Detailed |

**(1) Monitoring microbial diversity, composition, and function**

High-throughput amplicon sequencing is an increasingly common method used to characterise microbial diversity and community composition in a sample. Amplicon-based data can be used to assess differences in microbial communities across restoration treatments, controls, or ages (Fig. 4). Since amplicon sequencing is increasingly accessible and affordable, there has been rapid, recent growth in restoration studies using this approach (Mohr *et al.*, 2022). This method presents a detailed picture of microbial diversity and community composition, which is not provided by culture-dependent methods or phospholipid fatty acid analysis (PLFA) approaches. Since phospholipids are only collected from live microbes during sampling, PLFA provides a snapshot of live microbial biomass. As such, PLFA has an advantage over DNA sequence-based approaches where DNA is sampled from both live and dead microbes and living biomass cannot be estimated (Seymour, 2019). However, unlike sequence-based approaches, PLFA cannot provide detailed taxonomic insight into microbial diversity or composition. Therefore, it has been recommended that combining PLFA and sequence-based approaches can provide an accurate assessment of both live microbial biomass and community composition (Nkongolo & Narendrula-Kotha, 2020a).

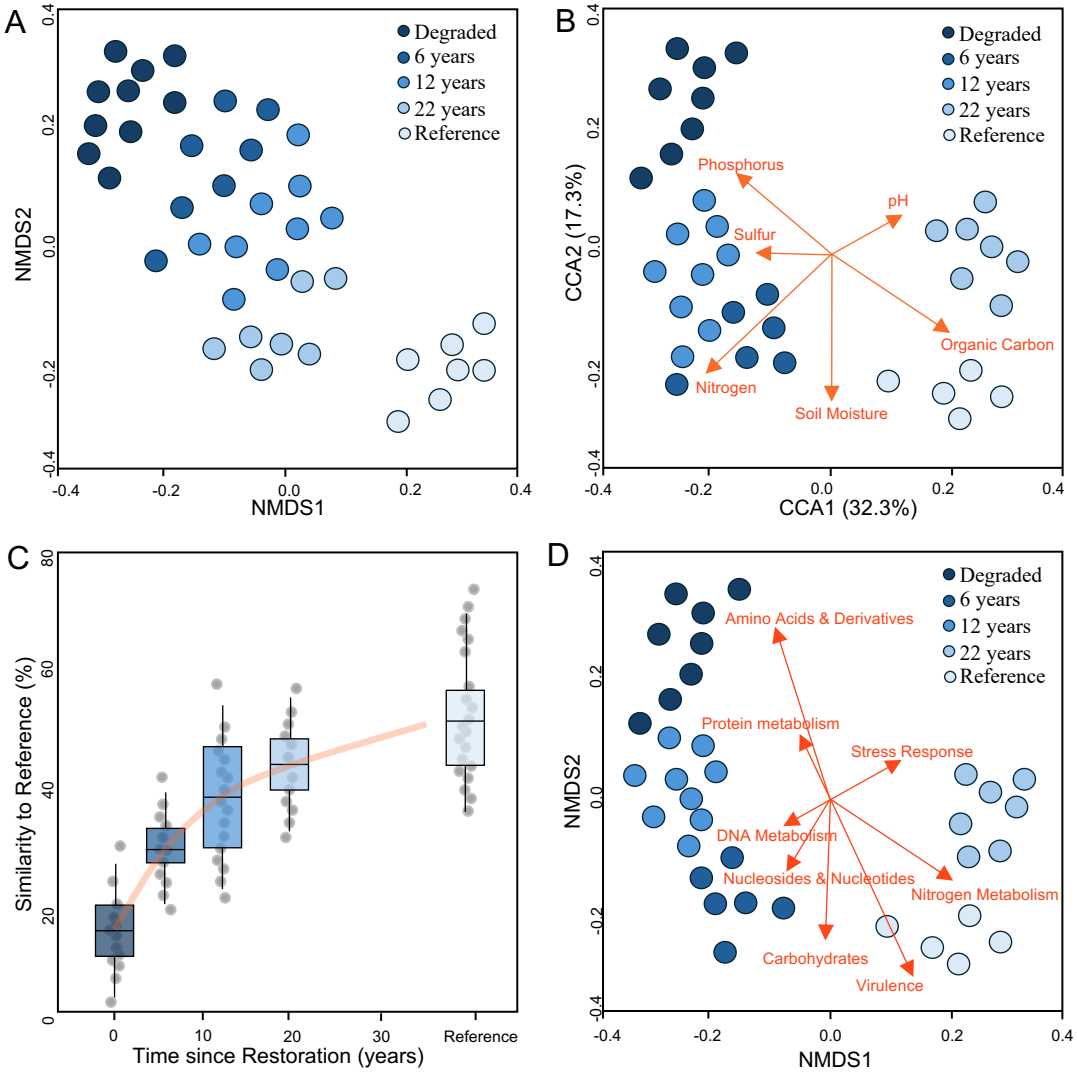


***Fig. 4.*** *Illustrations of examples of data presentations useful for monitoring soil microbiota in restoration. (A) A non-metric multidimensional scaling (NMDS) ordination from amplicon sequencing data comparing differences in microbial community composition across a restoration chronosequence. The ordination indicates communities become increasingly similar to Reference sites with increasing time since restoration. (B) A constrained correspondence analysis (CCA) ordination indicating how microbial community composition is constrained by soil physical and chemical properties. (C) Boxplots indicating the similarity to reference sites of microbial communities across a restoration chronosequence. The box plots indicate increased similarity to reference sites with increased time since restoration and the red line indicates the projected number of years until full recovery. (D) An NMDS ordination from shotgun metagenomic sequencing data showing shifts in*

*microbial functional gene composition across a restoration chronosequence overlayed with correlated Level 1 functional categories.*

Linking microbial amplicon sequencing data to microbial functions would be very useful for monitoring functional changes in soil microbiota during restoration. However, limited understanding of the functional roles of most microbial taxa, particularly in soils, presents a critical problem to the uptake of these tools (Morris, Meyer & Bohannan, 2020) and should be a key priority moving forward. Moreover, horizontal gene transfer between taxa would further confound amplicon-based taxonomy and functionality linkages, and as such, inferring function from amplicon data is not currently advised (Morris *et al.*, 2020; Makiola *et al.*, 2020). Some bioinformatic tools can estimate functionality from amplicon sequence data, such as PICRUSt2 (Douglas *et al.*, 2020) and Tax4Fun2 (Wemheuer *et al.*, 2020). However, there are many limitations with these approaches including that predicted functions represent approximations (not exact matches to the reference functions), and functional predictions are biased towards existing reference genomes and less likely to provide good functional characterisations for understudied environments such as soil (Douglas *et al.*, 2020; Sun, Jones & Fodor, 2020).

Growing opportunities from shotgun metagenomic and/or metatranscriptomic data sets are now available to provide high-quality insights into microbial functions in a restoration context (Breed *et al.*, 2019; Sun *et al.*, 2020). Shotgun metagenomics is similar to amplicon sequencing, but instead of amplifying a targeted gene region, it involves random sequencing of all DNA from within a sample. These random fragments of the metagenome within a sample can be aligned to functional and taxonomic databases and/or assembled into metagenome-assembled genomes

(MAGs). These approaches can provide functional gene abundance data directly instead of just taxonomic data or inferred functions from amplicon data. Restoration scientists can then interrogate these functional gene abundance data for functions of interest – such as genes associated with nitrogen fixation or primary productivity – and compare these before and after restoration to assess changes to key ecological functions and processes (Sun & Badgley, 2019). Importantly though, metagenomics involves a much higher sequencing cost, and the complexity of data processing and analysis requires expertise that could place a disproportionate burden on restoration projects. Furthermore, both amplicon and shotgun metagenomics do not discern between active and inactive organisms as relic DNA in the sample is also sequenced (Li *et al.*, 2017; Sun & Ge, 2023).

An additional layer of functional information can be obtained by collecting, isolating, and sequencing RNA (as opposed to DNA used in amplicon and metagenomic approaches) from a soil sample with metatranscriptomics. This technique can be a powerful asset in studying soil ecosystem services carried out by microbiota but is not yet widely used in ecological contexts (Breed *et al.*, 2019). Analysing total community RNA transcripts can potentially reveal a microbiome's gene expression under specific conditions, known as the active functional profile. This approach provides an opportunity to study direct alterations of the (meta-)transcriptome in response to different environmental conditions. High functional redundancy is common in soil microbiomes (Louca *et al.*, 2018; Prosser, 2020) and identifying relationships between microbial community structure and function remains challenging because observed community functions are often difficult to link to specific taxonomic groups. Furthermore, RNA-based methods are generally more

expensive and time-consuming than DNA-based methods (Cordier *et al.*, 2019), and the unstable nature of RNA molecules presents a technical challenge. Because transcriptional profiles can vary considerably over time, any information gained *via* metatranscriptomics should be interpreted as a 'snapshot' in time. Nonetheless, metatranscriptomics can be a powerful asset in trying to shed light on the dynamics of ecosystem functions carried out by microbiota and warrants consideration as part of a multi-omics approach (Aguiar-Pulido *et al.*, 2016).

**(2) Monitoring restoration trajectories with soil microbiota**

Due to their essential roles in ecological processes and rapid responses to environmental changes, soil microbiota are increasingly recognised for their use as bioindicators of recovery following ecosystem restoration (Coban *et al.*, 2022; Rawat *et al.*, 2022). Changes in land use, vegetation composition, and soil physical and chemical properties can lead to reciprocal changes in soil microbiota (Delgado-Baquerizo *et al.*, 2020). Accordingly, microbiota depend on feedbacks with aboveground ecosystem components and monitoring changes to microbiota following restoration interventions can present a holistic indication of any potential recovery trajectory, or lack thereof (van der Heyde *et al.*, 2022).

Using chronosequence-based amplicon sequence data, Liddicoat *et al.* (2022) modelled trajectories of soil microbiota recovery towards reference states (Fig. 4B). The authors assessed whole-soil bacterial communities and included multiple reference samples, and measures of similarity among these, to determine the level of natural variation that should be recognised when setting realistic targets for rehabilitation. Analysis of the entire community avoids reliance on any specific

indicator taxa – whose presence and abundance may vary considerably with site history and current conditions. Asymptotic logarithmic models (i.e. the response variable approaches a given value with a diminishing rate of change as the independent variable increases) were used to visualise trends and predict recovery timeframes as similarity-to-reference values increased with rehabilitation age, approaching a nominal target defined as the median of among-reference similarities.

Moving beyond community composition-based metrics alone, Sun & Badgley (2019) used shotgun metagenomics to assess changes in both community composition and functional gene abundance across a mine site restoration chronosequence (Fig. 4C, D). The metagenomic data indicated taxonomic shifts with restored samples becoming more like the reference with increasing age as well as successional patterns in important functional groups associated with nitrate and ammonia oxidisers. The authors did however highlight that while relative abundances of methanotrophs and methane monooxygenase genes increased through the chronosequence, their levels were still much lower than those from unmined reference sites even 31 years post revegetation.

As ongoing knowledge gaps are addressed and further refinements are made to how we effectively incorporate microbiota in restoration, ensuring benefits from the use of these tools become equitable is essential to ensure society and nature benefit equally across the globe. The use of many of the tools described here and collection and dissemination of data is already skewed away from the global south. For example, the US National Center for Biotechnology Information (NCBI) nucleotide database contains no sequence-read archives from restoration studies in South

America, Africa, or much of Asia (aside from China and Japan) (Robinson *et al.*, 2023). Securing adequate funding for restoration is already a barrier to restoration success and further reliance on costly technologies to effect positive outcomes will likely further increase the restoration gap between developed and developing nations. Ensuring equitable benefit from improved integration of soil microbiota is likely a more difficult task than overcoming technical barriers or knowledge gaps but is essential to effecting improvements where they are most needed.

# V. CONCLUSIONS

(1) Better integration of soil microbiota into restoration planning, interventions and monitoring has clear potential to improve ecosystem restoration outcomes.

(2) The research community should tackle several key knowledge gaps to help integrate soil microbiota into ecosystem restoration, including: (*i*) how do we best determine causation in microbial responses to restoration, (*ii*) when is the inclusion of soil microbiota worthwhile, (*iii*) what do *'good'* soil microbial communities look like and what elements can be generalised across broad restoration settings, (*iv*) what methods of inoculating restoration sites with whole microbial communities are most effective and how do we limit disturbance to remnant habitat while sourcing inoculants, (*v*) what barriers impede the establishment of targeted microbiota such as plant growth-promoting rhizobacteria and how do we overcome them, and (*vi*) how do we ensure equitable access to these tools to benefit society and nature maximally across the globe?

(3) Further collaboration between practitioners and cross-disciplinary researchers can help to address knowledge gaps, increase our understanding of restoration-

focused microbial processes and apply established microbiota-focused restoration techniques and knowledge to practical on-the-ground applications.

(4) Experiments with spatial and temporal components embedded into restoration projects that account for confounding factors will help overcome the limitations of observational studies in an effective way to fill knowledge gaps. Modelling techniques – including structural–causal models – will be useful for developing causal understanding and informing research priorities.

(5) Whole-soil translocations and microbial inoculations can drive successional changes in both soil microbiota and aboveground vegetation towards a target reference ecosystem; further research however is needed to understand barriers in microbial establishment and how to overcome them. To limit further degradation associated with sourcing soil inocula, these inoculations can be scaled up using various seed-enhancement technologies that add specific microbial inoculants to seeds as a coating or extruded pellets.

(6) Positive soil legacies can be promoted in restoration sites to prime soil microbiota to provide a fitness advantage to future plant generations and instil greater resiliency to environmental stressors including climate change-associated increases in aridity.

(7) Advancements in various ’omics technologies allow for effective monitoring of recovery trajectories to assess progress following restoration and continually adapt management strategies based on progress. Recovery of both microbial community composition and their ecologically important functions should be investigated to determine the state of the ecosystem and any further interventions needed to affect the restoration of biodiverse and functional ecosystems.

# VI. ACKNOWLEDGEMENTS

This work was supported by the Australian Research Council (grant numbers LP190100051, LP190100484, DP210101932) and the New Zealand Ministry of Business Innovation and Employment (grant UOWX2101). This project was supported by the Holsworth Wildlife Research Endowment and the Ecological Society of Australia. We thank two anonymous reviewers for their substantial effort in providing extensive comments that have improved our manuscript.

## VII. AUTHOR CONTRIBUTIONS

S.D.P., R.J.H., R.J.B., C.D.W., S.L.K. and M.F.B. conceived the study. S.D.P., R.J.H., R.J.B., S.L.K. and M.F.B. wrote the first draft of the paper with input from all authors.

# Chapter 2:

# Agricultural land-use legacies affect soil bacterial communities following restoration in a global biodiversity hotspot

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The candidate was the primary author of the manuscript, conducted the majority of the work, and led all aspects of the project. Conceptualisation and project design: **SDP**, SLK, AS, MFB. Field work and data generation: **SDP**, CCD, AS. Formal analysis: **SDP**, CL, MFB. Manuscript writing – original draft: **SDP**. All authors contributed to revisions and editing and approved the final submission. This chapter has been published in *Biological Conservation*.

Throughout this chapter I use the pronoun *'we'* to acknowledge the contributions of my coauthors, following the customary etiquette in published manuscripts.

Published in *Biological Conservation* (https://doi.org/10.1016/j.biocon.2023.110437)

Biological Conservation 290 (2024) 110437

Contents lists available at ScienceDirect

Biological Conservation

journal homepage: www.elsevier.com/locate/biocon

# Agricultural land-use legacies affect soil bacterial communities following restoration in a global biodiversity hotspot

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ARTICLE INFO

*Keywords:*
Land-use change
Microbiota
Post-agricultural restoration
Soil legacies

ABSTRACT

Ecosystem restoration in post-agricultural landscapes is a critical response to agricultural land abandonment, climate change, and the escalating biodiversity crisis. However, effective restoration of these landscapes can be hampered by land-use legacies that create biotic and abiotic barriers to ecosystem recovery, particularly in ancient Tertiary landscapes where vegetation is adapted to nutrient deficient soils. While our understanding of how to overcome these barriers when restoring plant communities is improving, there is limited knowledge of how these legacies impact on recovery of soil microbiota – the biodiverse and functionally-important communities of soil microbes. Here, we used amplicon sequencing of the bacterial 16S rRNA gene extracted from soils across a restoration project in southwest Western Australia, a global biodiversity hotspot, to examine recovery of soil microbiota following post-agricultural restoration. We sampled soils at six sites under four land conditions – degraded post-agriculture, actively revegetated post-agriculture, passively regenerated, and remnant bushland – generating 1,609,618 sequences corresponding to 15,009 unique bacterial taxa. We show that soil bacterial communities in revegetated and degraded samples were similar across sites but strongly dissimilar to adjoining remnant samples. We show that limited recovery of bacterial communities was linked to elevated soil phosphorus levels. Together, our results indicate soil microbiota have not recovered despite revegetation taking place up to 17 years ago, and this lack of recovery is likely driven by soil nutrient legacies from past agricultural practices. Our study highlights a key challenge faced by conservation practitioners when integrating soil microbiota into ecosystem restoration in post-agricultural landscapes in ancient, nutrient-poor landscapes.

## 1. Introduction

The conversion of natural ecosystems for agriculture is a principal cause of global terrestrial biodiversity declines (Foley et al., 2005; Leclère et al., 2020). As demand for agriculture increases, so does the need to conserve biodiversity to maintain vital ecological support systems (Cramer et al., 2008). The land area needed to meet the demand of agricultural production is still expanding, although this expansion is now outpaced by agricultural land abandonment driven largely by biophysical (e.g., decreased productivity, climate change, accessibility) and socioeconomic (e.g., migration, declining profit) factors (Poore, 2016; Subedi et al., 2022).

The restoration of post-agricultural landscapes back into functional and biodiverse native ecosystems presents an opportunity to ease – or even reverse – global biodiversity declines (Chazdon et al., 2020). However, persistent agricultural land-use legacies can pose significant challenges to the restoration of pre-disturbance ecological communities both above and below ground (Parkhurst et al., 2022a; Standish et al., 2006; Turley et al., 2020). These land-use legacies can be particularly impactful in old Tertiary landscapes, such as southwestern Australia, in contrast to younger recently glaciated landscapes found across the northern hemisphere that can be quicker to recover following agricultural abandonment (Cramer et al., 2008). These old landscapes are highly weathered and nutrient impoverished and are particularly sensitive to overcoming agricultural legacies stemming from the addition of fertilisers and other land management procedures that substantially

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https://doi.org/10.1016/j.biocon.2023.110437
Received 29 October 2023; Received in revised form 20 December 2023; Accepted 22 December 2023
Available online 8 January 2024

# Abstract

Ecosystem restoration in post-agricultural landscapes is a critical response to agricultural land abandonment, climate change, and the escalating biodiversity crisis. However, effective restoration of these landscapes can be hampered by land-use legacies that create biotic and abiotic barriers to ecosystem recovery, particularly in ancient Tertiary landscapes where vegetation is adapted to nutrient deficient soils. While our understanding of how to overcome these barriers when restoring plant communities is improving, there is limited knowledge of how these legacies impact on recovery of soil microbiota – the biodiverse and functionally-important communities of soil microbes. Here, we used amplicon sequencing of the bacterial 16S rRNA gene extracted from soils across a restoration project in southwest Western Australia, a global biodiversity hotspot, to examine recovery of soil microbiota following post-agricultural restoration. We sampled soils at six sites under four land conditions – degraded post-agriculture, actively revegetated post-agriculture, passively regenerated, and remnant bushland – generating 1,609,618 sequences corresponding to 15,009 unique bacterial taxa. We show that soil bacterial communities in revegetated and degraded samples were similar across sites but strongly dissimilar to adjoining remnant samples. We show that limited recovery of bacterial communities was linked to elevated soil phosphorus levels. Together, our results indicate soil microbiota have not recovered despite revegetation taking place up to 17 years ago, and this lack of recovery is likely driven by soil nutrient legacies from past agricultural practices. Our study highlights a key challenge faced by conservation practitioners when integrating soil microbiota into ecosystem restoration in post-agricultural landscapes in ancient, nutrient-poor landscapes.

**Key words:** land-use change, microbiota, post-agricultural restoration, soil legacies

# 1. Introduction

The conversion of natural ecosystems for agriculture is a principal cause of global terrestrial biodiversity declines (Foley *et al.*, 2005; Leclère *et al.*, 2020). As demand for agriculture increases, so does the need to conserve biodiversity to maintain vital ecological support systems (Cramer *et al.*, 2008). The land area needed to meet the demand of agricultural production is still expanding, although this expansion is now outpaced by agricultural land abandonment driven largely by biophysical (e.g., decreased productivity, climate change, accessibility) and socioeconomic (e.g., migration, declining profit) factors (Poore, 2016; Subedi, Kristiansen & Cacho, 2022).

The restoration of post-agricultural landscapes back into functional and biodiverse native ecosystems presents an opportunity to ease – or even reverse – global biodiversity declines (Chazdon *et al.*, 2020). However, persistent agricultural land-use legacies can pose significant challenges to the restoration of pre-disturbance ecological communities both above and below ground (Parkhurst *et al.*, 2022a; Standish *et al.*, 2006; Turley *et al.*, 2020). These land-use legacies can be particularly impactful in old Tertiary landscapes, such as southwestern Australia, in contrast to younger recently glaciated landscapes found across the northern hemisphere that can be quicker to recover following agricultural abandonment (Cramer *et al.*, 2008). These old landscapes are highly weathered and nutrient impoverished and are particularly sensitive to overcoming agricultural legacies stemming from the addition of fertilisers and other land management procedures that substantially alter ecosystem properties (Hopper & Gioia, 2004; Standish *et al.*, 2006).

Land-use conversion for agriculture alters multiple ecosystem components and these changes can persist long-term – potentially for millennia – following the cessation of agricultural practice (Brudvig *et al.*, 2021). Animal grazing and tillage lead to soil compaction, fertiliser application to increase productivity alters natural soil nutrient levels, cropping and frequent disturbance introduces persistent exotic plant species, and the removal and fragmentation of native vegetation depletes soil seed banks and limits the recovery of dispersal-limited flora (Flinn & Vellend, 2005; Cramer *et al.*, 2008; Murphy *et al.*, 2004). All these legacies present challenges not only for the restoration of aboveground biota, but also belowground soil microbiota (Turley *et al.*, 2020). Soil microbiota are also susceptible to land-use change and, as vegetation complexity and soil abiotic properties are altered, so too are soil microbiota (Fierer, 2017). While the revegetation of biodiverse native flora is the typical focus of post-agricultural restoration, soil microbiota are only recently being considered in a restoration context (Coban *et al.*, 2022; Mohr *et al.*, 2022). Research to date on the recovery of ecologically important soil microbiota following restoration of post-agricultural landscapes is limited (Watson *et al.*, 2022), and effectively absent in the context of the southwest Australian floristic region, a recognised global biodiversity hotspot containing old, climatically buffered, infertile landscapes (OCBILs) (Hopper, Silveira & Fiedler, 2016).

While flora and fauna are often the primary focus in ecosystem restoration, soils are home to an estimated 59% of all life on earth (Anthony *et al.*, 2023). Soil microbiota form some of the most biodiverse ecosystem components and contribute to a fundamental range of ecological processes including nutrient cycling, decomposition, and ecosystem productivity (Fierer, 2017; Harris, 2009). While our understanding of

the drivers of soil microbial diversity and composition has improved greatly in the past decade (Mohr et al 2022), the response of soil microbiota to traditional revegetation is still uncertain and varies across restoration settings (Watson *et al.*, 2022). The development of soil eDNA metagenomic applications to characterise environmental microbiota has seen a focus on observational post-restoration research to assess the recovery of soil microbiota following revegetation (e.g., (Liddicoat *et al.*, 2022; Schmid *et al.*, 2020; Sun *et al.*, 2021). While these studies often indicate a trajectory towards recovery, recovery is often incomplete and confounded by the restoration methods applied and the nature of the original disturbance (Watson *et al.*, 2022; Lem *et al.*, 2022; Liddicoat *et al.*, 2022). Numerous microbiota-focussed post-mining restoration assessments indicate that – even with the substantial disturbance of surface strip mining and transfer of topsoil – microbiota composition often progresses towards a reference like composition following restoration efforts (van der Heyde *et al.*, 2020; Peddle *et al.*, 2022; Liddicoat *et al.*, 2022). However, the recovery of microbiota following restoration of post-agricultural land in global biodiversity hotspots has received very little attention and it is unclear to what extent post-agricultural soil legacies persist and impede recovery of soil microbiota in this context.

The diversity and composition of soil bacteria are shaped by multiple ecosystem properties (McBratney, Mendonça Santos & Minasny, 2003). While there is more evidence for above- and below-ground relationships for fungi than bacteria due to, for example, the direct symbiotic relationship that mycorrhizal fungi have with plants, there are associations between aboveground alpha diversity and soil bacterial alpha diversity (Steinauer *et al.*, 2015; Wang *et al.*, 2022; Eisenhauer *et al.*, 2017b).

However, the relationship between aboveground plant alpha diversity and soil bacterial community composition (beta diversity) is stronger than the alpha diversity associations (Prober *et al.*, 2015; Tedersoo *et al.*, 2016; Fierer, 2017). Strong evidence also exists for the effects of soil abiotic properties, particularly soil pH, organic carbon, and nutrient levels, on soil bacterial diversity and composition (Delgado-Baquerizo & Eldridge, 2019; Fierer, 2017). Accordingly, there is often an expectation that the restoration of a highly biodiverse vegetation community will initiate concomitant changes in soil bacterial communities (Harris, 2009; Breed *et al.*, 2019; Watson *et al.*, 2022).

To assess these expected changes in soil bacterial communities following revegetation, we sampled soils from four land conditions across six post-agricultural restoration sites to examine the recovery of soil bacterial communities in a post-agricultural setting in the southwest Australian floristic region – a global biodiversity hotspot. We hypothesised that: (1) sites that have undergone active revegetation of biodiverse native flora will have bacterial communities that more closely resemble those from uncleared remnant sites than sites that are still degraded because the revegetation can facilitate a combined recovery in soil bacteria; (2) sites with an agricultural land-use history will have soil physicochemical legacies that will be different to those from sites with no agricultural land-use history, and these post-agriculture sites will have soil properties that associate with changes in soil bacterial community communities.

# 2. Materials and Methods

## 2.1 Site description and soil sampling

This study was conducted in the southwest Australian floristic region, a global biodiversity hotspot with exceptional levels of plant species richness, endemism, and habitat fragmentation from land clearing (Myers *et al.*, 2000; Hopper & Gioia, 2004). Here, we sampled soils from six sites in September 2020 between the Stirling Range National Park and Fitzgerald River National Park (Yarraweyah Falls, Monjebup Reserve, Red Moort Reserve, Chingarrup Sanctuary, Chereninup Reserve, and Beringa Reserve; Fig 1). All six sites are currently managed for conservation purposes and were previously agricultural properties. The region is punctuated with expanses of old, climatically buffered, infertile landscapes (OCBILs) which, since the early Cretaceous, have sustained persistent weathering without continental climactic extremes and are generally nutrient poor, and particularly low in phosphorous (Hopper *et al.*, 2016).

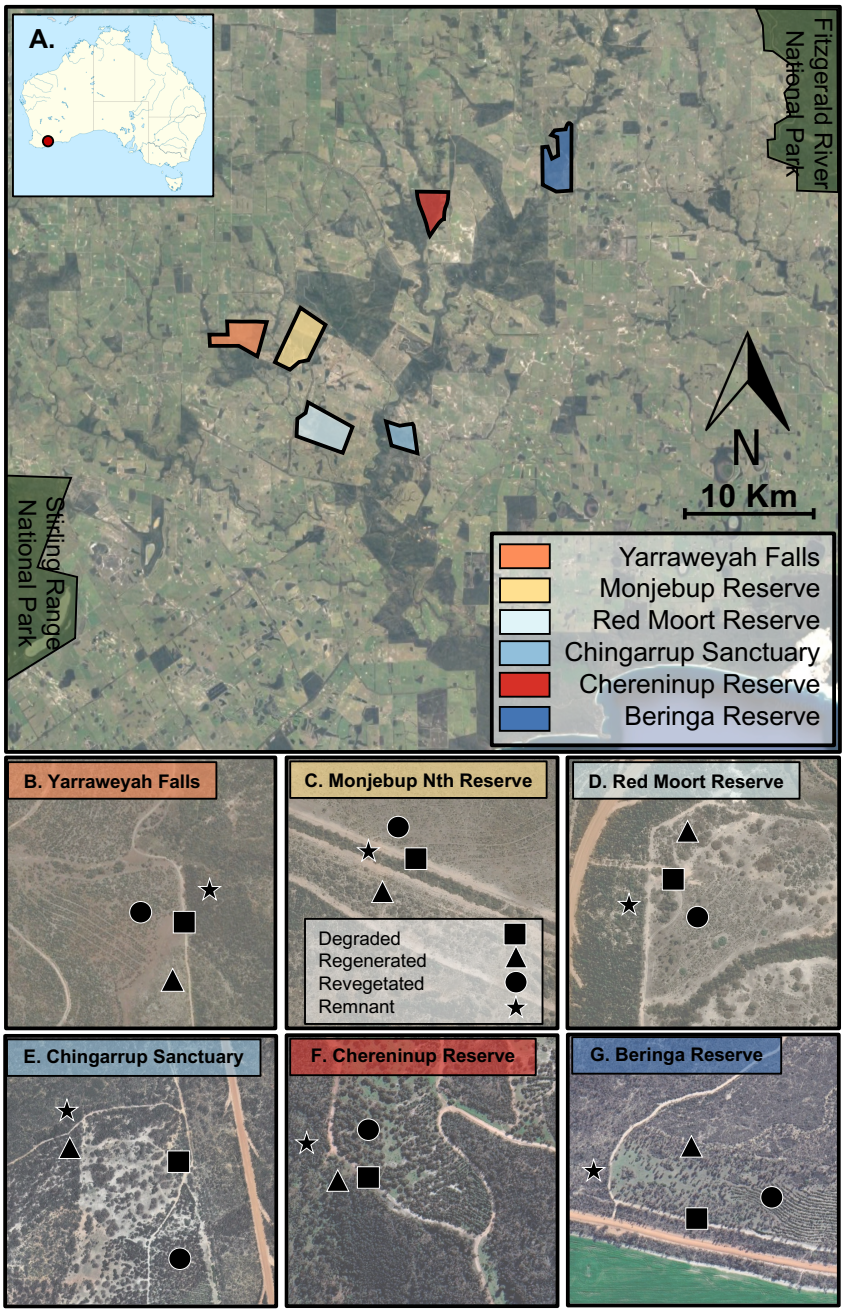


***Figure 1. Map of study area.*** *Maps that show (A) location of the six study sites used for soil sampling in southwest Western Australia; and sampling design and layout of degraded, passive regenerated, revegetated, and remnant land conditions at each sampled site (B) Yarraweyah Falls, (C) Monjebup North Reserve, (D) Red Moort Reserve, (E) Chingarrup Sanctuary, (F) Chereninup Reserve, (G) Beringa Reserve.*

Land clearing – predominantly for crop-based agriculture – has resulted in substantial native vegetation fragmentation. However, multiple conservation focused groups have been returning ecological connectivity via revegetating a heterogenous, highly biodiverse mallee heath vegetation community as part of the Gondwana Link project (Bradby *et al.*, 2016). The region has a Mediterranean-type climate with hot, dry summers and cool, wet winters and average annual rainfall of 460mm (Australian Bureau of Meteorology 2023). Soils in the region are typically nutrient poor and range from shallow to deep sandy duplexes with sandy topsoil over clay or heavy textured subsoils (van Gool, Stuart-Street & Tille, 2018).

Each of our six sites contain areas where native vegetation was cleared for agricultural cropping and grazing in the mid-1900s but has since undergone active native revegetation between 2003 and 2018. Revegetation methods consist of direct seeding a species-rich mix of local provenances as described in Jonson (2010). Each site contained areas of (i) remnant natural vegetation that were not historically cleared or subjected to agriculture (hereafter Remnant), (ii) areas that were historically cleared with little to no agricultural use that were passively regenerated (no direct seeding or planting, hereafter Regenerated), (iii) areas that were historically cleared and used for predominantly cropping agriculture that have since been actively revegetated through direct seeding (hereafter Revegetated), (iv) degraded areas that were historically cleared for agriculture and currently undergo active management to suppress vegetation for the purpose of firebreaks or access tracks (hereafter Degraded) (Fig S1).

All six sites contained these four land conditions in close proximity (mean distance between land conditions at each site range between 88 m at Chereninup Reserve and 228 m at Chingarup Sanctuary). For each land condition at each site, we established a 25 x 25 m plot and systematically collected nine soil subsamples from the top 10 cm of topsoil to capture plot heterogeneity. These nine subsamples were then pooled and homogenised in a sterile plastic bag, and a 50 mL sample collected and frozen on-site for subsequent DNA extraction and sequencing (described below) at the Australian Genome Research Facility (Adelaide, South Australia), and a 500 g sample collected for physicochemical analysis at CSBP Laboratories (Perth, Western Australia) quantifying phosphorus (Colwell), potassium (Colwell), sulphur (KCl 40), organic carbon (Walkley-Black), nitrate nitrogen, ammonium nitrogen, electrical conductivity, pH (water), pH (CaCl2), and physical characteristics.

**2.2 eDNA sequencing and bioinformatics**

DNA was extracted from each soil sample using the Qiagen DNeasy PowerLyzer PowerSoil Kit following manufacturer's instructions and quantified fluorometrically. Soil bacterial 16S rRNA was amplified using the 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) primer set before 300 base pair paired end sequencing on the Illumina MiSeq platform. Diversity profiling was conducted using QIIME 2 2019.7 (Bolyen *et al.*, 2019). Primers were trimmed and demultiplexed raw reads were quality filtered using the cutadapt plugin and denoised with DADA2 (Callahan *et al.*, 2016) to zero-radius operational taxonomic units (zOTUs). Taxonomy was assigned to zOTUs using the q2-feature-classifier (Bokulich *et al.*, 2018) classify-sklearn naïve Bayes taxonomy classifier. zOTUs not

classified as “Bacteria” or classified as “Bacteria_unclassified” at the phylum level were discarded, along with those classified as “Mitochondria” or “Chloroplast”.

### 2.3 Data analysis

R version 4.3.1 (R Core Team, 2023) was used for all statistical analyses. Rarefaction curves were generated comparing observed zOTU richness and sample sequence read depth to assess if sample zOTU richness was adequately represented by read depth and to determine an appropriate read depth for rarefaction to ensure unbiased comparisons across samples (Fig S2). All samples were determined to have adequate sequence read depth and all samples were rarefied to 22,427 reads to match the sample with the lowest read depth using the *rarefy_even_depth* function with *Phyloseq* (McMurdie & Holmes, 2013).

### 2.4 Bacterial alpha diversity and community composition

We calculated observed and estimated chao1 bacterial zOTU richness as well as Simpson and Shannon diversity indices using *Phyloseq* and compared these metrics across our four land conditions and six sites using permuted analysis of variance with the *aovp* function in *lmperm* v2.1.0 (Wheeler, Torchiano & Torchiano, 2016) with 999 permutations.

Bacterial community compositions across our four land conditions and six sites were visualised using principal coordinates analysis (PCoA) ordinations of Bray-Curtis distances from the rarefied zOTU abundances using *ordinate* in *phyloseq*. Differences in bacterial community composition across land conditions and sites were assessed using permuted multivariate analysis of variance (PERMANOVA)

implemented with the *adonis2* method in *vegan (Oksanen et al., 2013)*. Homogeneity of group dispersions was tested with the *betadisper* function in *vegan*.

To further evaluate the similarities of bacterial communities between sites, we used Bray-Curtis distances to calculate similarity values (i.e., 100%*(1 – distance)) for each sample to all remnant samples, including each remnant sample to all other remnant samples (Liddicoat et al. 2022). The distribution of similarity to remnant values across the four land conditions were then displayed as boxplots. A Kruskal-Wallis multiple comparison test was used to determine whether the 'similarity to remnant' values differed across our land conditions. Significant pairwise differences between land conditions were then identified using post-hoc Dunn tests with Bonferroni correction to adjust *p* values for multiple comparisons. We also calculated a 'site-adjusted similarity to remnant' index to account for site-specific effects. To do this, we calculated the similarity to remnant value of each degraded, regenerated, and revegetated sample only to its paired site-specific remnant sample and displayed these values in boxplots. We explored land condition effects with a Kruskal-Wallis test. To explore if bacterial communities in revegetated and passively regenerated samples were becoming more like those in remnant samples with increasing time since revegetation and time since change in land-use, we calculated similarity to remnant values separately for both revegetated and passively regenerated samples. We plotted these values against the number of years since revegetation for the revegetated samples (Chereninup Reserve revegetated in 2003 = 17 years, Chingarrup Sanctuary revegetated in 2005 = 15 years, Yarraweyah Falls revegetated in 2013 = 7 years, Monjebup Reserve revegetated in 2014 = 6 years, Red Moort Reserve revegetated in 2015 = 5 years, and Beringa Reserve

revegetated in 2018 = 2 years) and the number of years since change in land-use (Beringa Reserve cleared in 1993 = 27 years since change in land use, Chereninup Reserve cleared in 1987 = 33 years since change in land use, Monjebup Reserve cleared in 1986 = 34 years since change in land use, Yarraweyah Falls cleared in 1975 = 45 years since change in land use, Chingarrup Sanctuary cleared in 1970 = 50 years since change in land use, and Red Moort Reserve also cleared in 1970 = 50 years since change in land use) for the passively regenerated samples.

Heatmaps of the relative abundances of all bacterial phyla, class, and order from non-rarefied zOTU data created with the *plot_heatmap()* function in *phyloseq* were used to visualise if any particular taxa were driving changes in community composition across the land conditions. A stack plot was generated using *ggplot2 v3.4.4* to visualise phylum level rarefied bacterial relative abundance of all samples grouped by the four land conditions. For each of the ten most abundant bacterial phyla, we assessed differences in relative abundances across land conditions using permuted ANOVA with the *aovp* function in *lmperm*.

**2.5 Soil physicochemical associations**

Associations between bacterial community composition and scaled (i.e., mean-centred and divided by the standard deviation) soil chemical variables were visualised and assessed using constrained correspondence analysis (CCA) with the *cca()* function in *vegan*. Highly correlated (>0.75) variables were identified and removed from our analysis using the *findcorrelation* function in *caret* (Kuhn, 2015). Remaining variables (= total vegetation cover, tree cover, grass cover, ammonium nitrogen, nitrate nitrogen, phosphorus (Colwell), sulphur, organic carbon, pH (H2O),

copper, manganese, zinc, aluminium, and sodium) were used for automated model selection by permutation tests with the *ordistep* function in *vegan*. The model selected variables – sodium, manganese, phosphorus (Colwell), and sulphur – and their association with bacterial community composition was visualised with a CCA and tested with a permuted ANOVA with 999 permutations. Differences for each soil physicochemical variable across land conditions were visualised with boxplots and differences tested using analysis of variance with the *aov* function.

To further explore correlations between key soil physicochemical variables and bacterial community composition, we used the PCoA coordinates of each sample and calculated the Euclidean distance of each sample to the remnant centroid. We then plotted these Euclidean distances against the phosphorus, pH, iron, manganese, sodium, sulphur, and organic carbon value for each sample. Each of these variables were either selected in our CCA model or were expected to strongly associate with bacterial community composition (Fierer, 2017). Correlations were assessed for all chosen variables and the distance to remnant centroid with the *cor.test* function using the *spearman* method.

**2.6 Spatial autocorrelation**

We explored associations between bacterial community composition (using Bray-Curtis ecological distances) and geographic distances between samples to test for the presence of spatial autocorrelation. Here, we used Haversine distance matrices with the *distm* function in *geosphere* (Hijmans *et al.*, 2017), which calculates the distance between every sample based on a spherical land surface from GPS coordinates. The relationship between the Haversine distance matrix and Bray-Curtis

distance matrix was examined with a Mantel test in *vegan* using the *spearman* method with 9,999 permutations.

# 3. Results

We sequenced 16S rRNA amplicons from the 24 soil samples collected across our six sites, which generated a total of 1,609,618 bacterial 16S rRNA reads. We identified 15,009 unique bacterial zOTUs with a mean of 67,067 (± 20,831 SD) sequence reads per sample (Table 1). Following quality filtering including removal of taxa not assigned as bacteria or those assigned as chloroplast or mitochondria and rarefaction to the lowest sample read depth of 22,427 reads, 13,964 zOTUs remained for analysis.

***Table 1.*** *Mean (± SD) bacterial amplicon sequence variant (zOTU) abundance for the four land conditions.*

| Land Condition | Samples (n) | zOTU abundance (±SD) |
|---|---|---|
| **Degraded** | 6 | 77,765 ± 17,797.53 |
| **Regenerated** | 6 | 51,061 ± 24,052.23 |
| **Revegetated** | 6 | 65,815 ± 21,798.52 |
| **Remnant** | 6 | 73,627 ± 11,290.65 |

## 3.1 Bacterial alpha diversity and community composition

Land condition and site had significant effects on bacterial community composition (Fig. 2a; PERMANOVA: condition $df = 3$, $F = 1.578$, $p = 0.004$; site $df = 5$, $F = 1.978$, $p = 0.001$). As samples from one site (Chereninup) were strongly differentiated from the rest, we also ran PCoA and PERMANOVA analyses excluding this site. Land condition and site still had significant effects on bacterial community composition

(Fig. 2b; PERMANOVA: condition *df* = 3, *F* = 1.944, *p* = 0.002; site *df* = 4, *F* = 1.516, *p* = 0.014). Neither land condition nor site influenced bacterial alpha diversity (Table 2, Fig. S3: ANOVA, *p* > 0.05 for all).

***Table 2.*** *Mean (± SD) bacterial amplicon sequence variant (zOTU) Observed and Chao1 richness and Shannon and Simpson diversities from four land conditions across six restoration sites in southwest Western Australia.*

| **Land Condition** | **Samples (n)** | **zOTU Richness (±SD)** | | **Diversity (±SD)** | |
|---|---|---|---|---|---|
| | | **Observed** | ***Chao 1*** | ***Shannon*** | ***Simpson*** |
| **Degraded** | 6 | 1402.8 ±289.0 | 1445.7 ±302.9 | 6.46 ±0.29 | 0.994 ±0.0026 |
| **Regenerated** | 6 | 1008.0 ±371.5 | 1031.9 ±398.4 | 6.19 ±0.38 | 0.995 ±0.0030 |
| **Revegetated** | 6 | 1167.2 ±442.4 | 1195.7 ±459.4 | 6.24 ±0.40 | 0.997 ±0.0009 |
| **Remnant** | 6 | 1274.5 ±130.9 | 1308.7 ±136.1 | 6.41 ±0.14 | 0.995 ±0.0029 |
| ***p* value** | | 0.234 | 0.236 | 0.403 | 0.570 |

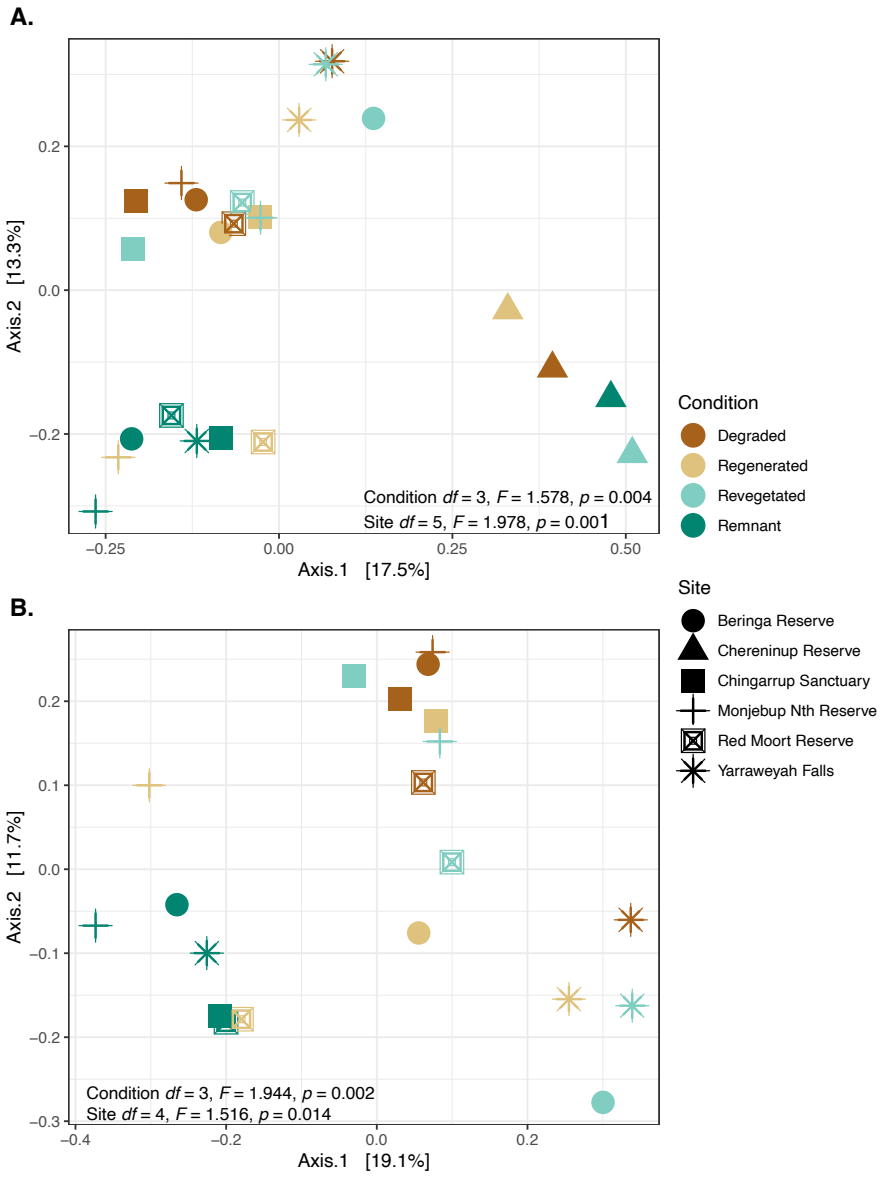


***Figure 2.*** *Principal Coordinate Analysis (PCoA) ordinations of Bray-Curtis distances indicating bacterial community composition across four land conditions (Degraded, Passive Regenerated, Revegetated, and Remnant) at six sample sites. (A) Data from all samples from all sites, which indicates that a single site (Chereninup Reserve) is explaining the most variation in bacterial community composition, but remnant samples are also similar to each other but different to degraded and revegetated sites. (B) Bacterial community composition for the sites remaining after excluding Chereninup, which indicates bacterial communities are different across land conditions with both Degraded and Revegetated samples similar to each other but different to Remnant sites.*

Land condition had a significant effect on similarity to remnant values (Kruskal-Wallis: $p = 0.022$), with revegetated and remnant land condition samples differing significantly (Fig. 3a: Dunn post-hoc test with Bonferroni correction: $p = 0.019$). Land condition also had a significant effect on site-adjusted similarity to remnant values (Fig. 3b: $p = 0.009$), where revegetated sites had lower values than passive regenerated sites (Fig. 3b: Dunn post-hoc test with Bonferroni correction: $p = 0.008$). Although we detected an effect of time since revegetation on similarity to reference values (Fig. 3c: Kruskal-Wallis, $p = 0.013$), we found no indication of a trajectory towards recovery with increasing time since revegetation. Time since change in land-use did not have an effect on similarity to reference among passive regeneration samples (Fig. 3d: Kruskal-Wallis, $p = 0.155$).

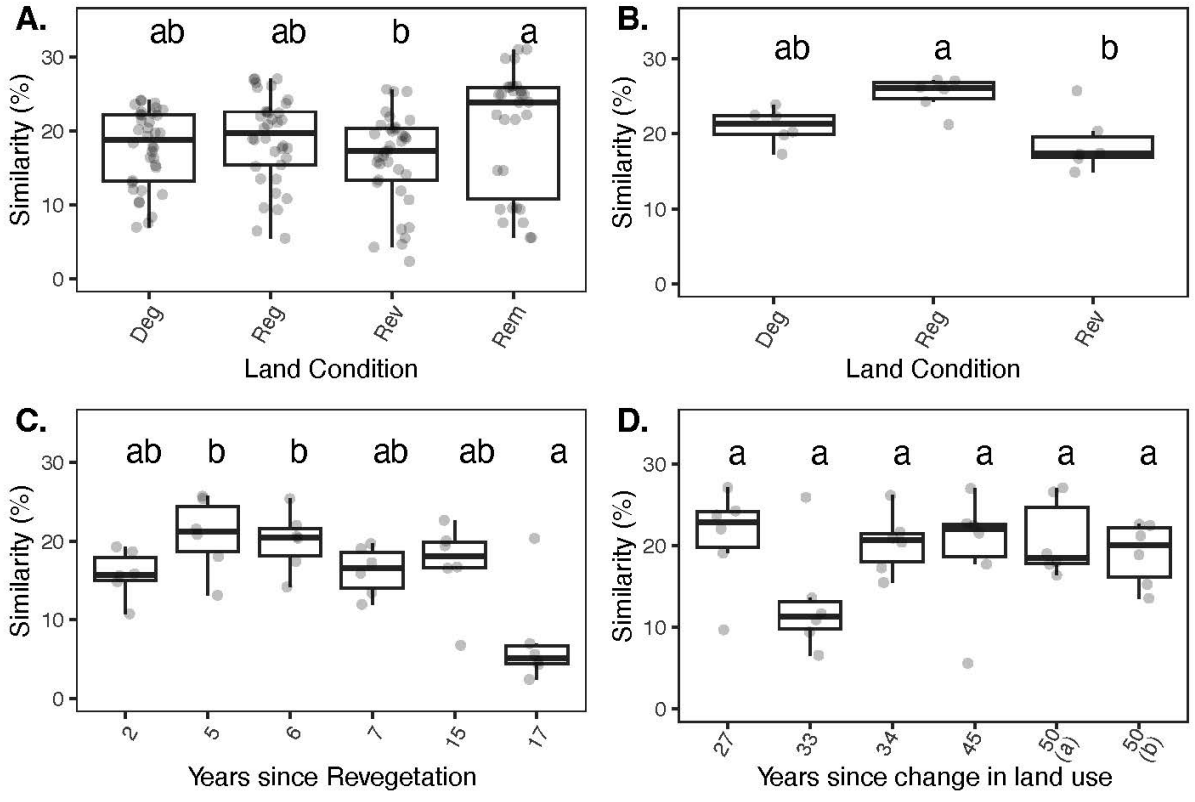


***Figure 3.*** *Boxplots indicating similarities of Bray-Curtis distance to remnants to assess how similar bacterial communities are compared by (A) land condition (Deg =*

*Degraded, Reg = Regenerated, Rev = Revegetated, and Rem = Remnant) against all remnant samples; (B) land condition against site specific remnants (i.e., similarity of each degraded, passive regenerated, and revegetated sample to their sites paired remnant sample); (C) years since revegetation for all revegetated samples; (D) years since change in land-use (the number of years since vegetation was last cleared) for all passively regenerated samples. Groups not sharing a letter are significantly different ($p<0.05$) as indicated by Kruskal-Wallis multiple comparison tests with Dunn post hoc and Bonferroni correction.*

### 3.2 Soil physicochemical associations

Manganese, sodium, phosphorus, and sulphur each associated with bacterial community composition, largely explained by high levels of these variables at each land condition at Chereninup (Fig. 4a). After removing Chereninup, iron, sodium, sulphur, and phosphorus had effects on bacterial community composition (Fig. 4b). Increased levels of iron and decreased levels of phosphorus associated with bacterial communities in all remnant samples, and increased levels of sodium and sulphur associated with bacterial communities in remnant and passive regenerated samples from both Monjebup North Reserve and Red Moort Reserve. Increased phosphorus associated with bacterial communities in degraded, passive regenerated, and revegetated samples from Yarraweyah Falls and the passive regenerated sample from Beringa Reserve.

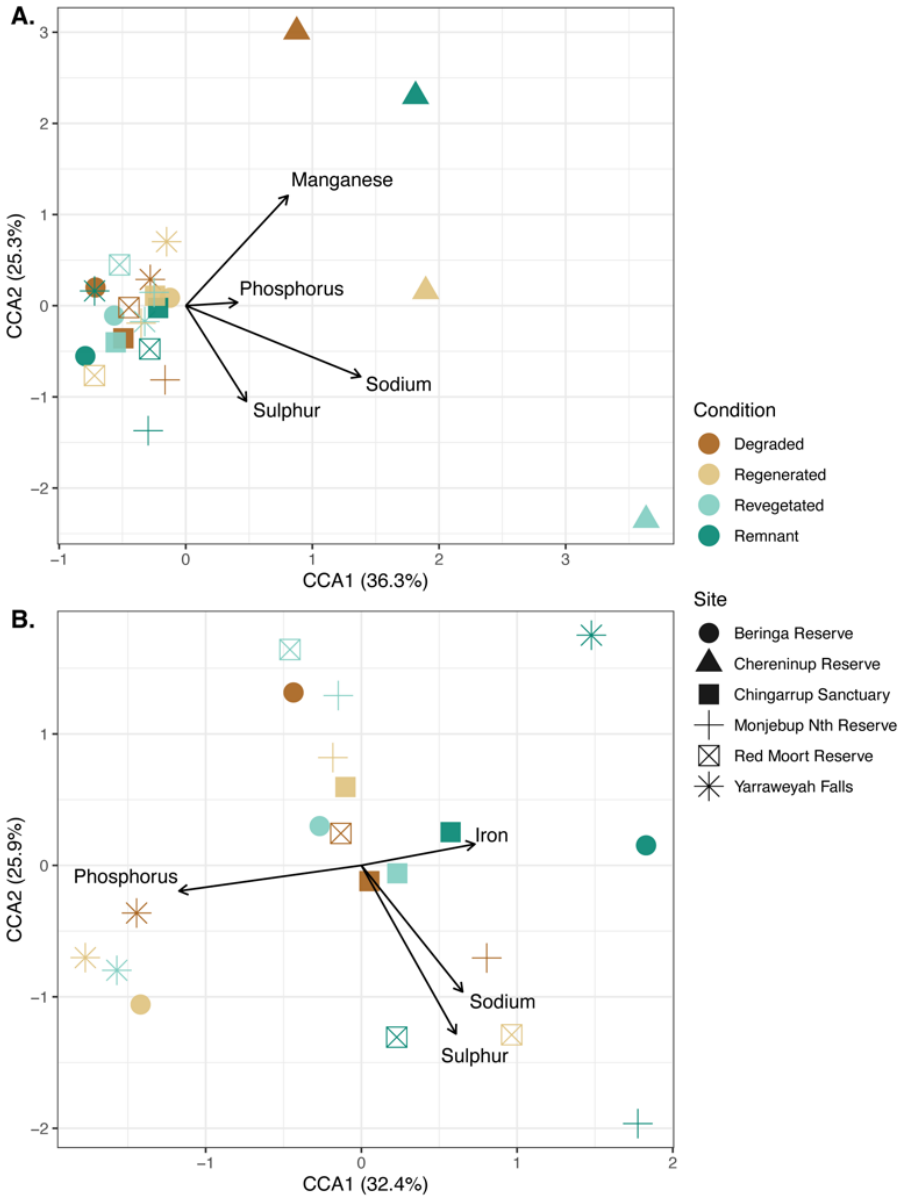


***Figure 4.*** *Constrained correspondence analysis (CCA) ordinations of bacterial community composition from Bray Curtis distances and associated soil physicochemical properties for (A) all samples and sites with manganese, phosphorus (Colwell), sodium, and sulphur associating with bacterial community composition at a single site (Chereninup Reserve); and (B) with Chereninup Reserve removed from analysis to explore associations in all other samples. Increased levels of iron, sodium, and sulphur are associating with community composition in mainly remnant sites and increased phosphorus levels are associated with degraded, passive regenerated, and revegetated samples from Yarraweyah Falls and a passive regenerated sample from Beringa Reserve.*

We found a strong positive correlation between the Euclidean distance of all samples to the remnant centroid and sample phosphorus levels (Fig. 5: spearman, $r$ = 0.621; $p$ = 0.001) and a positive correlation between distance to remnant centroid and manganese (Fig. 5: spearman, $r$ = 0.456, $p$ = 0.025). Iron was negatively correlated with distance to remnant centroid (Fig. 5: spearman, $r$ = -0.44, $p$ = 0.031). Soil physicochemical variables were not significantly different across land conditions (Fig. S4: ANOVA, $p$ > 0.05 in each case).

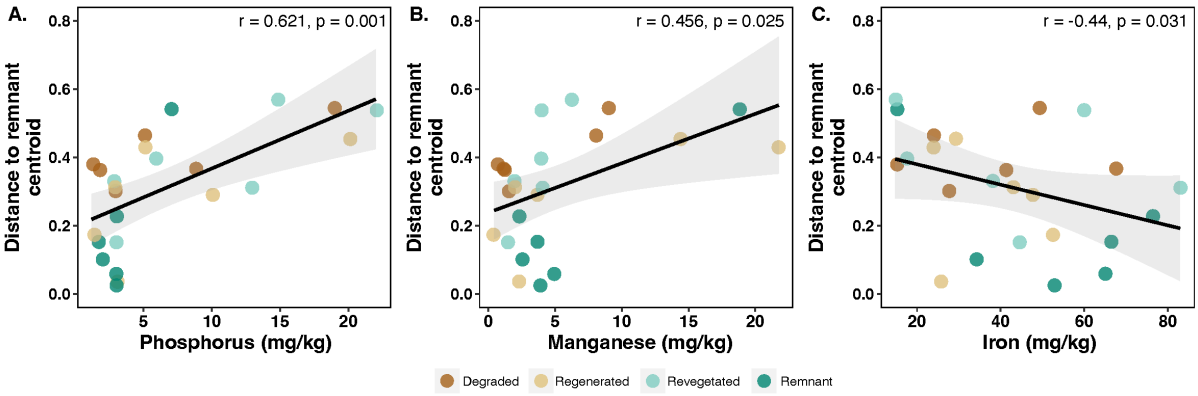


***Figure 5.*** *Correlation plots for the Euclidean distance of each sample to the remnant centroid compared against (A) phosphorus, (B) manganese, and (C) iron. Phosphorus and manganese each displayed positive correlations with the distance to the remnant centroid, and iron displayed a negative correlation with the distance to remnant centroid.*

### 3.3 Relative abundance of bacterial phyla

The ten most common bacterial phyla from all samples represented 98.56% of the total bacterial relative abundance, with the remaining phyla grouped as 'other minor phyla'. Land condition only had an effect on the relative abundance of the phylum Proteobacteria (Fig. S5: permuted ANOVA, $p$ = 0.001) which was most abundant in remnant samples, followed by passive regenerated samples, degraded samples, then revegetated samples.

### 3.4 Spatial autocorrelation

Bacterial community composition and geographic distance between sample locations were not correlated (Fig. S6: Mantel, $r = 0.073$, $p = 0.164$).

## 4. Discussion

Our assessment of soil bacterial communities following post-agricultural restoration revealed a surprising lack of recovery. Sites that were actively revegetated up to 17 years prior to sampling were similar to long-standing degraded sites, where vegetation has been actively suppressed since the cessation of agricultural activities. However, bacterial communities in degraded and revegetated samples differed strongly from those in uncleared remnant bushland sites, with no patterns of increasing similarity to remnants with increasing time since revegetation. Our results contrast to many previous studies that found soil bacterial recovery following revegetation, including a global meta-analysis (Watson *et al.*, 2022), Western Australian post-mining contexts (Liddicoat *et al.*, 2022; van der Heyde *et al.*, 2022), and northern hemisphere post-agricultural contexts (Barber *et al.*, 2017; Barber *et al.*, 2023). Sites with an agricultural land-use legacy of elevated phosphorus levels had bacterial communities that were less similar to those in remnant sites, indicating that soil phosphorus was a likely abiotic barrier to this recovery.

### 4.1 Bacterial communities have not recovered following revegetation

Our restoration sites showed little recovery in soil bacterial communities towards their spatially-paired remnant sites. This lack of recovery contrasts with the increasing amount of research that largely shows recovery trajectories following

revegetation efforts. Globally, soil bacterial communities in revegetated sites tend to become increasingly like reference sites with increasing time since revegetation (Watson *et al.*, 2022), and signs of recovery have been seen in as little as three years following revegetation (Peddle *et al.*, 2022). Previous research on post-mining restoration in southwest Western Australia has also presented strong evidence of soil bacterial community recovery following revegetation, with similarity to reference states achieved from 40-60 years (van der Heyde *et al.*, 2020; Liddicoat *et al.*, 2022). Microbiota also appear to recover quickly in geologically younger northern hemisphere soils. For example, Barber *et al.* (2017) and Turley *et al.* (2020) reported patterns consistent with successional recovery of soil microbiota following post-agricultural restoration. In our study, the site with the longest time since revegetation (17 years) not only showed no indication of recovery, but also had the lowest similarity to remnant value of all our revegetated sites. Previous research assessing recovery of bacterial communities over time has indicated time since revegetation is a crucial factor for recovery in both post-agricultural (Barber *et al.*, 2017; Barber *et al.*, 2023; Lem *et al.*, 2022) and post-mining systems (Liddicoat *et al.*, 2022; Ngugi *et al.*, 2018; van der Heyde *et al.*, 2020), with bacterial communities in revegetated sites becoming more like the reference sites over time. In contrast, bacterial community recovery at our study sites appears to be inhibited by abiotic (incl. soil phosphorus levels) and biotic (e.g., established agricultural niche-adapted bacterial communities) barriers stemming from agricultural land-use legacies. While long term agricultural land-use is known to alter soil biotic and abiotic properties (Brudvig *et al.*, 2021; Cramer *et al.*, 2008; Parkhurst *et al.*, 2022b), in old infertile landscapes, such as old, climatically buffered, infertile, landscapes (OCBILs) (Hopper & Gioia, 2004),

our results suggest these soil legacies may present more substantial barriers to recovery than they do in geologically younger, more fertile landscapes.

Soil bacterial communities within remnant or reference sites often display considerable spatial variation (Peddle *et al.*, 2022; Liddicoat *et al.*, 2022), which impacts comparisons made between reference sites and other land conditions, including sites that are being restored. It is imperative to account for this variation when assessing progress towards restoration targets (Brudvig *et al.*, 2017; Hobbs & Norton, 1996; White & Walker, 1997). To account for this variation among remnant sites, we assessed the similarities of each of our degraded, passively regenerated, and revegetated samples only to their site-specific paired remnant sample. When we did this, we found that soil bacterial composition in revegetated samples had the lowest similarity to their paired site-specific remnant values, and passively regenerated samples displayed the highest similarity to remnant values. While this higher similarity to remnant value could suggest that passive recovery may return bacterial communities that more closely resemble natural targets, the extent of agricultural land-use at some of our passively regenerated sample locations was unclear. It is likely that the higher similarity to remnant values in the passively regenerated samples resulted from lower levels of degrading processes and/or abiotic barriers to recovery (e.g., long-term application of fertiliser, elevated levels of phosphorus).

### 4.2 Agricultural legacies and their association with bacterial communities

We show that elevated soil phosphorus was likely a major barrier to the recovery of soil bacterial communities. This result is consistent with previous work that has

shown elevated phosphorus to be a barrier to biodiverse plant community establishment in OCBILs (Lambers *et al.*, 2011). Agricultural legacies – including elevated levels of phosphorus – in our sites appear to be inducing a stable alternative state of soil bacterial communities and given our results, is unlikely to shift towards a recovery state with additional time alone. This study adds to a list of known impacts in addressing elevated soil phosphorus in restoration efforts. While previous research has addressed this elevated soil phosphorus barrier in native seed viability, seed sourcing, and seed germination contexts (Standish & Hobbs, 2010), additional work (e.g., soil microbiota focused restoration interventions) is required to improve recovery of soil biota in these post-agricultural settings in ancient landscapes.

The soil bacterial community was particularly different at one of our sites (Chereninup), which was largely explained by different levels of manganese, phosphorus, sodium, and sulphur across all four land conditions at this site (incl. the remnant) as shown in the CCA. This indicates site-specific variation in soil properties displayed at Chereninup was not necessarily a result of agricultural land-use legacy. However, when we ran our analyses without samples from Chereninup, soil abiotic properties associated strongly with different bacterial communities across the four land conditions. In particular, bacterial communities in remnant soils associated with lower levels of phosphorus and higher levels of iron. Soils in southwest Western Australia are phosphorus limited due to ancient weathering, particularly in OCBILs (Hopper & Gioia, 2004). These processes have led to many locally-derived adaptations in the diverse flora of the region to efficiently mine phosphorous in these phosphorus-limited soils (Lambers *et al.*, 2011). Therefore, enduring phosphorous

impacts on soil bacterial communities are likely to inhibit recovery following revegetation when post-agricultural legacies are not adequately addressed during restoration interventions. Our results support others that have also shown that bacterial communities are impacted by altered soil phosphorus levels (Oliverio *et al.*, 2020; Leff *et al.*, 2015).

Ironstone gravels, arising from ancient weathering, also feature in soil profiles within the southwest Australian floristic region (Hopper *et al.*, 2016; van Gool *et al.*, 2018). Land and soil disturbance for agriculture has potential to alter the distribution and bioavailability of iron in soil profiles, for example, via vegetation clearance, tillage, erosion, plant nutrient removal, and altered soil moisture retention. The availability of iron shapes soil microbiota (Jin, Ye & Zheng, 2013), so altered soil iron availability may represent a further source of post-agricultural land-use legacies impacting on soil bacterial communities.

# 5. Conclusions

We report results from a global biodiversity hotspot that go against the global trend of soil bacterial community recovery following aboveground revegetation. We conclude that agricultural land-use legacies were likely inhibiting this recovery. It is well-known that ecological restoration of aboveground biota within ancient infertile landscapes is a major challenge, however our findings are the first to show that this challenge also applies to the functionally important and highly biodiverse belowground soil bacterial communities. Accordingly, more research is required to develop strategies that help overcome post-agricultural land-use legacies that act above- and below-ground. Improving post-agricultural restoration prospects for soil

microbiota will also benefit aboveground outcomes and help ameliorate continuing issues for the successful establishment of aboveground communities. While interventions such as whole soil inoculations may be promising, further research is needed to determine the most effective methods for establishing whole microbial communities and whether or not such inoculations can even overcome existing soil legacies in degraded landscapes.

## CRediT authorship contribution statement:

**SDP:** Conceptualisation, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review and editing. **CCD:** Validation, Resources, Writing – review and editing. **SLK:** Investigation, Resources, Writing – review and editing, Supervision. **AS:** Conceptualisation, Investigation, Resources, Writing – review and editing. **MFB:** Conceptualisation, Methodology, Investigation, Formal analysis, Writing – review and editing, Supervision.

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## Acknowledgements

The authors wish to thank all landholders – Bill and Jane Thompson, Eddy and Donna Wajon, and Bush Heritage Australia – for their generosity and assistance with accessing sites used in this study. We thank Rachel Standish and Tina Parkhurst for comments on a draft version of the manuscript, and to Michelle Hall, Jaclyn Smith, and Alex Hams for assistance with soil sampling, field work, or other on-site and

project logistics. We acknowledge and pay respect to the Traditional Owners of the land on which this study was conducted: the Minang and Goreng people of the Noongar Nation.

## Funding

This study was funded by a philanthropic donation from Peter and Maxine Wilshaw administered through Bush Heritage Australia. The funding source had no role in establishing the study design or in data analysis but did provide on ground support in selecting sampling sites that met the study requirements and helped provide specific context in interpreting results from a land management perspective. SDP was supported with an Australian Government Research Training Program Stipend and the Holsworth Wildlife Research Endowment administered by the Ecological Society of Australia.

# Chapter 3:

# Soil microbial functions associate with persistent agricultural legacies and indicate an alternative stable state following restoration plantings in a global biodiversity hotspot

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This chapter has been prepared for submission to *Restoration Ecology*. A preprint is available on *Authorea*: https://doi.org/10.22541/au.174301721.10879091/v1

The candidate was the primary author of the manuscript, conducted the majority of the work, and led all aspects of the project. Conceptualisation and project design: **SDP**, SLK, MFB. Field work and data generation: **SDP**, CCD. Formal analysis: **SDP**, RAE, CL, MFB. Manuscript writing – original draft: **SDP**. All authors contributed to revisions and editing and approved the inclusion of this chapter in my thesis.

Throughout this chapter I use the pronoun *'we'* to acknowledge the contributions of my coauthors, following the customary etiquette in published manuscripts.

## Abstract

The soil microbiome is a fundamental ecosystem component and research evaluating the recovery of microbial communities is increasingly used to monitor the efficacy of restoration efforts. However, given high levels of functional redundancy among soil microbial taxa and the subsequent lack of definitive taxa-function links, taxonomic assessments alone are unlikely to provide sufficient rigour to infer ecological recovery. Here, we applied shotgun metagenomics to soil samples collected across six post-agricultural restoration sites in southwest Western Australia to assess the recovery of microbial functions. We compared both taxonomic and functional diversity and composition across four land conditions: degraded, passive regenerated, revegetated, and remnant. We found no difference in the effective number of functions (alpha diversity) across land conditions. However, functional compositions (beta diversity) differed between remnant and revegetated conditions and associated with altered soil abiotic properties, especially available phosphorus. Remnant soils contained a higher effective number of phosphorus metabolism functions despite having the lowest levels of available phosphorus indicating their greater capacity for phosphorus acquisition and cycling using diverse pathways under nutrient-poor conditions. While our results show differences in total functional compositions, associations with post-agricultural soil abiotic legacies indicate functional adaptations to an alternative stable state rather than a lack of recovery or dysfunction. Together, our findings highlight that restoration interventions that directly target the soil microbiome (e.g., soil inoculation) are needed to facilitate recovery of the soil microbiome.

**Keywords**

alternative stable states, functional capacity, land-use legacy, metagenomics, microbiome, restoration, soil abiotic legacies, soil health

# 1. Introduction

The effective restoration of natural ecosystems on post-agricultural land is important to address global biodiversity declines (Chazdon *et al.*, 2020). However, post-agricultural restoration often faces numerous challenges arising from altered soil abiotic properties including homogenised soil structure from tillage practices and soil nutrient legacies from decades of fertiliser application (Brudvig *et al.*, 2021; Cramer *et al.*, 2008). These post-agricultural soil legacies can impede the recovery of both above- and below-ground ecosystem components despite concerted restoration efforts (Suding *et al.*, 2004; Turley *et al.*, 2020). Assessing the recovery of soil microbial communities and their ecological functions following restoration provides an informative monitoring tool for the effectiveness of restoration interventions (Robinson *et al.*, 2023; van der Heyde *et al.*, 2022).

Microbial communities show mixed responses to restoration plantings. Some studies indicate a lack of recovery in microbial communities decades after restoration interventions (Peddle *et al.*, 2024a; Turley *et al.*, 2020), while others more commonly indicate patterns towards recovery (Barber *et al.*, 2023; Liddicoat *et al.*, 2022), although recovery is often incomplete (Watson *et al.*, 2022). While assessing the recovery of soil bacterial communities following post-agricultural restoration sites in southwest Western Australia, Peddle *et al.* (2024a) showed that agricultural land-use legacies persisted up to 17 years after the cessation of agricultural practices. These land-use legacies also associated with a lack of recovery in soil bacterial community composition. While assessments of microbial communities can provide indications of ecological succession and recovery trajectories, a lack of recovery of community composition does not directly infer a lack of functional recovery due to high levels of

functional redundancy and horizontal gene transfer between taxa (Allison & Martiny, 2008). These high levels of functional redundancy and the inability to directly link function to taxonomy indicates that functional recovery could occur despite a lack of recovery of taxonomic composition. As such, more comprehensive meta-omics based methods that allow assessments of both microbial communities and their functional gene abundances are required to accurately assess the recovery of key microbially mediated ecological functions (Breed *et al.*, 2019; Robinson *et al.*, 2023).

Increasingly, studies are using single gene focussed amplicon or eDNA metabarcoding approaches to assess the recovery of microbial communities following restoration (Barber *et al.*, 2023; Mohr *et al.*, 2022). Integrating functional analyses into studies of the soil microbiome or soil health is crucial for understanding ecosystem processes (Raupp *et al.*, 2024), yet many studies primarily focus on taxonomic composition. Shotgun metagenomic approaches present an opportunity to enhance these analyses by enabling accurate assessments of microbial functional recovery, offering deeper insights into ecosystem recovery, resilience and biogeochemical cycling (Mason *et al.*, 2023; Singh *et al.*, 2024; Sun & Badgley, 2019).

Soil microbiota are fundamental components of healthy ecosystems, performing multiple simultaneous ecological functions relevant for nutrient cycling, primary productivity, and climate regulation (Delgado-Baquerizo *et al.*, 2016). Microbiota convert soil organic matter and facilitate mineral weathering (dissolution) of soil and rock substrates into bioavailable forms of nutrients (e.g., nitrogen, phosphorus) required by plants and other organisms (Cavicchioli *et al.*, 2019; Kleber *et al.*, 2015;

Samuels *et al.*, 2020). In phosphorus deficient soils, like those in southwest Western Australia, microbiota play crucial roles in the phosphorus cycle by mineralising organic phosphorus, solubilising inorganic phosphorus and facilitating plant uptake of phosphorus through symbiotic associations (Pang *et al.*, 2024). Furthermore, it is not possible to reliably predict functional contributions for these important roles, based solely on taxonomic identity (e.g., via amplicon or marker gene based sequencing approaches) (Nkongolo & Narendrula-Kotha, 2020b). However, shotgun metagenomic approaches that directly sequence and quantify microbial genomes allow more functionally-relevant assessments of crucial ecological processes in soil, such as nutrient cycling and organic matter decomposition (Sun & Badgley, 2019; Mason *et al.*, 2023; Robinson *et al.*, 2023). This is particularly valuable in restored ecosystems where shifts in microbial functional capacity – such as phosphorus metabolism – can indicate recovery trajectories and functional resilience (Peddle *et al.*, 2024b).

Here, we used shotgun metagenomics to characterise the diversity and composition of soil microbial communities and their functions across four land conditions (degraded, passive regenerated, revegetated, remnant) in six post-agricultural restoration sites in southwest Western Australia. Although previous amplicon-based assessments of the recovery of bacterial communities at these sites indicated a lack of recovery of community composition (Peddle *et al.*, 2024a), assessing the recovery of microbial functions – such as phosphorus metabolism – will be valuable for evaluating recovery trajectories, efficacy of restoration interventions, and any corresponding shifts in functional resilience (Mason *et al.*, 2023). We addressed the following research questions: *(i)* Has either active (i.e., our revegetated condition) or

passive (i.e., our regenerated condition) restoration led to the recovery of microbial functional diversity or composition? *(ii)* Do persistent agricultural land-use legacies associate with functional gene compositions across our four land conditions?

# 2. Materials and Methods

## 2.1 Site description and soil sampling

This study was conducted at six sites in southwest Western Australia (Yarraweyah Falls, Monjebup Reserve, Red Moort Reserve, Chingarrup Sanctuary, Chereninup Reserve, and Beringa Reserve), situated between the Stirling Range National Park and Fitzgerald River National Park (Figure 1). While all sites are currently managed for conservation, they were each previously used for agricultural cropping and experienced substantial clearing of native vegetation. The region falls within the southwest Australian floristic zone, recognised as a global biodiversity hotspot due to its extraordinary plant species richness, high levels of endemism, and extensive habitat fragmentation resulting from land clearing (Myers *et al.*, 2000; Gioia & Hopper, 2017). Land clearing, primarily for agricultural cropping, has resulted in highly fragmented native vegetation. To counteract this, various conservation groups are working to restore ecological connectivity by re-establishing a diverse mallee heath vegetation community as part of the Gondwana Link project (Bradby *et al.*, 2016). The region experiences a Mediterranean climate, with hot, dry summers and cool, wet winters, and receives an average annual rainfall of approximately 455 mm (Australian Bureau of Meteorology, 2025). The soils are typically nutrient-poor and range from shallow to deep sandy duplexes, characterised by sandy or sandy-loam topsoils overlaying clay or heavier-textured subsoils.

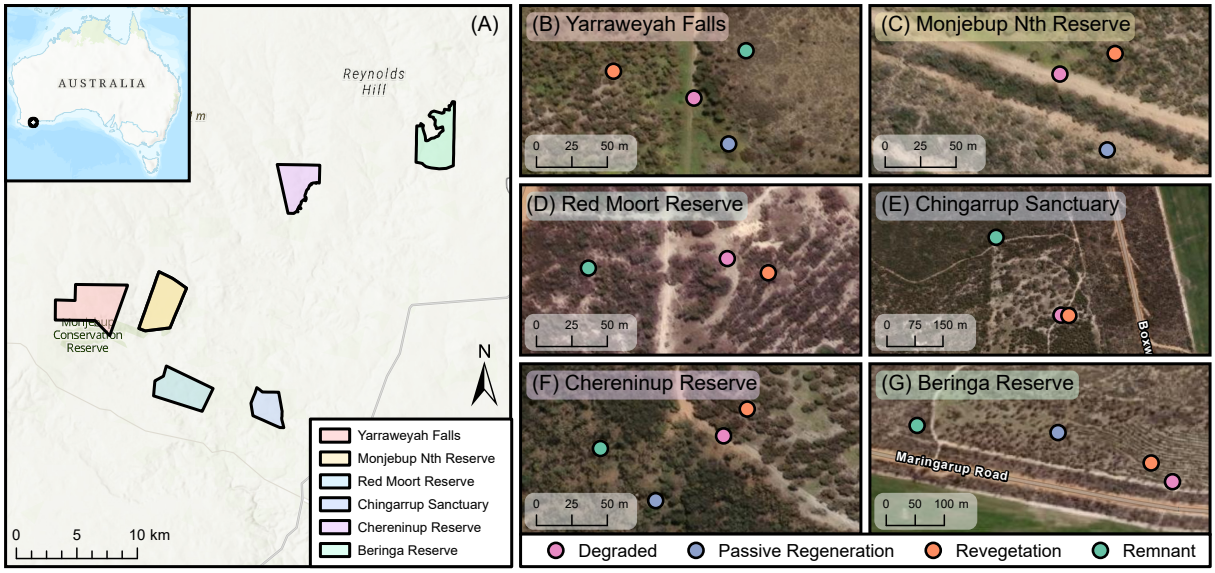


***Figure 1.*** *Maps indicating (A) location of the six study sites used for soil sampling in southwest Western Australia; and sampling design and layout of degraded, passive regeneration, revegetation, and remnant land conditions at each sampled site (B) Yarraweyah Falls, (C) Monjebup North Reserve, (D) Red Moort Reserve, (E) Chingarrup Sanctuary, (F) Chereninup Reserve, (G) Beringa Reserve*

Each of the six sites were largely cleared for agricultural cropping and grazing in the mid-20th century and have since undergone extensive revegetation between 2002 and 2017 using species-rich seed mixes sourced from local provenances. In this study, four distinct land conditions were identified within each site: (i) remnant natural vegetation that was never cleared or used for cultivation (hereafter, remnant), (ii) historically cleared areas with minimal to no agricultural use that passively regenerated without direct seeding or planting (hereafter, regenerated), (iii) formerly cleared agricultural land that has been actively revegetated via direct seeding (hereafter, revegetated), and (iv) degraded areas that were historically cleared for agriculture and are now actively managed to suppress vegetation for firebreaks or access tracks (hereafter, degraded). A sampling design was implemented to ensure each site contained all four land conditions in close proximity (Figure 1). At each site,

25 × 25 m quadrats were established within each of the four land conditions in September 2020. Within each of these quadrats, nine soil subsamples were systematically collected from the top 10 cm of topsoil to account for plot-scale spatial heterogeneity. These subsamples were pooled and homogenised, with a 50 ml sample frozen on-site for subsequent DNA extraction and 500 g of soil retained for physicochemical analysis at CSBP Laboratories (Perth, Western Australia) quantifying texture, pH, conductivity, organic carbon, available phosphorus (Colwell), ammonium nitrogen, nitrate nitrogen, potassium (Colwell), sulphur, and total copper, iron, manganese, zinc, aluminium, calcium, sodium, and boron.

**2.2 Shotgun metagenomic sequencing and bioinformatics**

DNA was extracted from each soil sample using the Qiagen DNeasy Powerlyzer Powersoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and quantified fluorometrically. Libraries were prepared using Nextera library prep kits (Illumina, San Diego, CA) then linear DNA libraries were converted into DNA nanoball (DNB) structures using the MGIEasy Universal Library Conversion Kit (MGI, China). Three of our samples failed library prep, and the remaining 21 libraries were sequenced at the South Australian Genomics Centre (SAGC) with the MGI DNBSEQ G400 (MGI, China) producing 2 x 150bp paired-end sequences. Bioinformatic processing of the metagenomics data was performed on the DeepThought high performance computing facility (Flinders University, 2021). Data cleaning was conducted using fastp v0.23.2 (Chen *et al.*, 2018), which included trimming adapters from DNB sequences. Taxonomic IDs were assigned using Kraken2 v2.0.7 (Wood, Lu & Langmead, 2019), followed by Bracken (Lu *et al.*, 2017) to estimate taxonomic abundances and then KrakenTools (Lu *et al.*, 2022) was used to create a taxonomic

abundance table. We assigned prokaryotic gene functions to the sequences (also termed reads) using SUPER-FOCUS v1.6 (Silva *et al.*, 2015) with the Diamond v0.9.14 aligner (Buchfink, Reuter & Drost, 2021). This functional assignment also grouped sequences into three thematic functional subsystems from the SEED database (Overbeek, Disz & Stevens, 2004).

### 2.3 Statistical analysis – Taxonomy

R version 4.4.0 (R Core Team, 2024) was used for all statistical analyses. Relative abundance normalisations were applied to both taxonomic and functional datasets to address differences in sequencing read depth across samples. As SUPER-FOCUS only assigns prokaryotic functions to reads, we removed any taxa assigned as eukaryote at the Kingdom level from our taxonomic dataset to match the prokaryote functional assignments of our functions dataset. To assess taxonomic alpha diversity, we calculated the effective number of species for each sample and tested for differences across land condition with ANOVA and Tukey post-hoc test using *vegan* (Oksanen *et al.*, 2013). Then, we visualised microbial community compositions across our land conditions and sites using non-metric multidimensional scaling (NMDS) ordinations based on Bray-Curtis distances using *ordinate* in the *phyloseq* (McMurdie & Holmes, 2013) package. We assessed differences in microbial compositions across land condition and site using permutational multivariate analysis of variance (PERMANOVA) using the *adonis2* method in *vegan*. Next, microbial community compositions and their associations with scaled (mean-centred and standardised) soil physicochemical variables were analysed and visualised using constrained correspondence analysis (CCA) with the *cca* function in *vegan*. Variables with a collinearity above 0.75 were removed using the

*findCorrelation* function in *caret* (Kuhn, 2015). The remaining variables underwent automated model selection with the *ordistep* function in *vegan*. Model-selected variables and their associations with bacterial and fungal composition were visualised in a CCA. The significance of these associations was assessed with permuted ANOVA with 999 permutations to determine whether the constrained ordination model explained a significant portion of community variation.

**2.4 Statistical analysis – Functions**

We first assessed alpha diversity of all functions in the dataset by calculating the effective number of functions (calculated as the exponent of Shannon's diversity index) (Jost, 2006) for each sample and comparing them across our four land conditions with ANOVA and Tukey post-hoc tests. Next, using identical methods outlined above, we visualised microbial functional compositions with an NMDS ordination and assessed differences in functional compositions across land condition and site with PERMANOVA. We then used CCA (methods detailed above) to assess associations in composition of all functions at the functional process level with soil physicochemical variables (methods detailed above). To inform further analyses of specific functional groups across the breadth of the functional dataset, we visualised the relative abundance of the top 20 most abundant levels of subsystem 1 for each sample with a stacked bar plot. We then tested for differences in the relative abundance of each subsystem 1 level across our four land conditions with permuted ANOVAs by performing individual analyses for each subsystem 1 group. Any level of subsystem 1 that differed across land condition was then retained for further analysis in addition to any subsystem 1 level linked to the abiotic legacies in these sites previously identified in Peddle *et al.* (2024a) (phosphorus metabolism, iron

acquisition and metabolism, sulphur acquisition and metabolism, and nitrogen metabolism).

### 2.5 Subsystem 1 functional groups

For phosphorus metabolism, iron acquisition and metabolism, sulphur acquisition and metabolism and any subsystem 1 level whose relative abundance differed across land condition, we then assessed alpha diversity, functional composition and associations with soil physicochemical properties, and the relative abundance of all subsystem 2 or 3 levels. For alpha diversity, we again calculated the effective number of functions for each sample and compared it across land condition with an ANOVA and Tukey post-hoc test using *vegan*. We assessed the composition of functions across land condition and site using NMDS ordinations and PERMANOVAs, and the associations of functional compositions with soil physicochemical properties across land condition using CCAs. We then assessed differences in the relative abundance of each subsystem 2 level (unless no subsystem 2 levels were assigned in which case we used subsystem 3 levels) with stacked bar plots and tested for differences in the relative abundance of each group individually across land condition with permuted ANOVAs. These analyses were repeated for all identified levels of subsystem 1. Furthermore, to assess which specific functions were driving differences in phosphorus metabolism, we used ANCOMBC2 on raw count data (Lin & Peddada, 2024) to evaluate pairwise differences in phosphorus metabolism functions at the function level in degraded, regenerated, and revegetated conditions all compared to the remnant (i.e., degraded-remnant, regenerated-remnant, and revegetated-remnant). Furthermore, for phosphorus metabolism functions we assessed the correlation between the

effective number of phosphorus functions and available soil phosphorus using Kendall's rank correlation test and visualised these data in a scatterplot.

# 3. Results

Our analysis generated taxonomic libraries with a total of 64,345,215 reads (mean 3,064,058 reads per sample) containing 13,940 unique microbial species (mean 10,344 per sample) (Table S1). Our functional libraries consisted of 72,657,207 reads (mean 3,459,867 per sample) containing 35,276 unique functions (mean 24,021 per sample) (Table S1).

## 3.1 Taxonomy

While our revegetated soil samples displayed the lowest values of effective number of species (mean effective no. species ± SD: revegetated 2196.32 ± 252.89, remnant 2419.69 ± 102.89, regenerated 2450.89 ± 95.28, degraded 2500.00 ± 265.11), the effective number of species did not differ across our four land conditions (Figure 2A, Table S2; ANOVA, $df = 3$, $F = 2.42$, $p = 0.102$). Microbial community composition, however, did vary across land condition and site (Table S3, Figure S1; PERMANOVA, condition, $df = 3$, $F = 2.24$, $p = 0.009$; site, $df = 5$, $F = 1.70$, $p = 0.029$). After removing co-linear variables, we found associations between microbial community composition and nitrate, pH, phosphorus, sulphur, aluminium, and iron (Figure 2B). We identified a total of 73 microbial phyla, with the 30 most abundant phyla representing 99.98% of phylum relative abundance. At phylum level, only the relative abundance Pseudomonadota differed across land condition (Figure S2A; Permuted ANOVA, $W = 12.89$, $p = 0.001$, *BH adj p* =0.03).

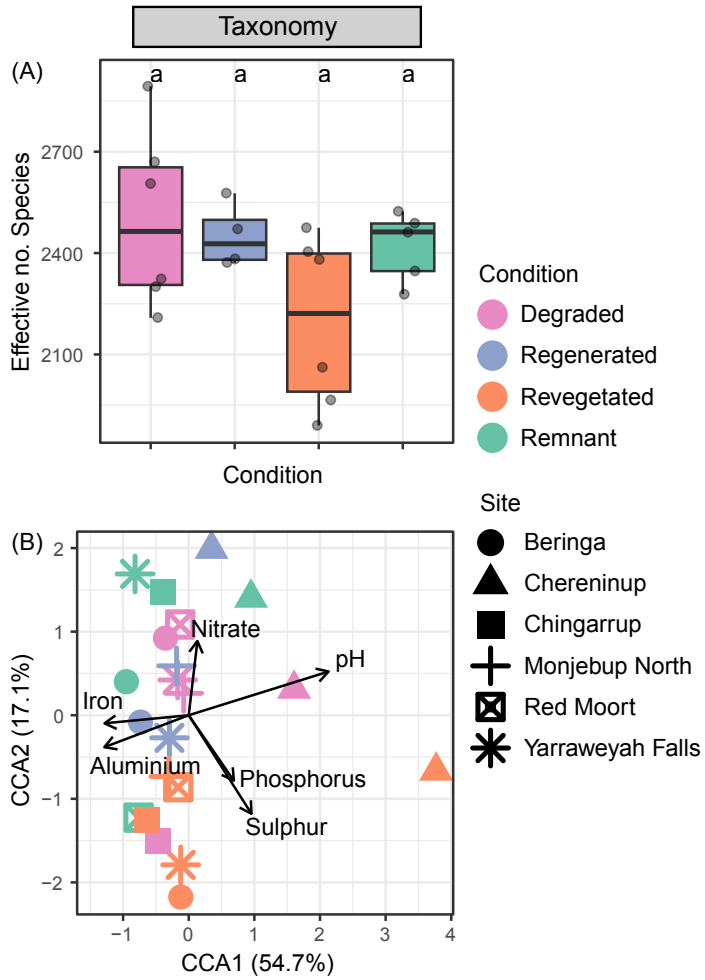


***Figure 2.*** *Taxonomic diversity, composition and associations with soil abiotic properties. (A) Effective number of species (alpha diversity) in soil samples collected across four land conditions. Groups sharing the same letter are not significantly different. (B) Constrained correspondence analysis (CCA) indicating microbial community composition in soil samples across four land conditions and the model selected soil abiotic properties that associate with those compositions.*

### 3.2 Functions

Across our full functional dataset, we found no difference in the effective number of functions between land conditions (Figure 3A; ANOVA, $p = 0.178$). However, we did find compositional differences when comparing all functions represented in samples across land condition and site (Table S3, Figure S1; PERMANOVA, condition $df = 3$, $F = 2.01$, $p = 0.015$; site $df = 5$, $F = 1.97$, $p = 0.006$), with remnant and revegetated

conditions displaying clear differences in communities. Our CCA also showed associations between functional compositions across land conditions and nitrate, pH, phosphorus and sulphur (Figure 3B).

We identified 35 functional groups (or themes) at the subsystem 1 level, the 20 most abundant of which represented 99.00% of the sum total functional capacity attributed across all samples (Figure S2). The relative abundance of amino acids and derivatives (permuted ANOVA, $W = 9.58$, *BH adj p* = 0.03), carbohydrates (permuted ANOVA, $W = 9.90$, *BH adj p* = 0.03), clustering-based subsystems (permuted ANOVA, $W = 11.23$, *BH adj p* = 0.02), DNA metabolism (permuted ANOVA, $W = 13.21$, *BH adj p* = 0.002), and regulation and cell signalling (permuted ANOVA, $W = 11.68$, *BH adj p* = 0.01) differed across land conditions (Figure S2) and therefore were examined in more detail (below), along with phosphorus metabolism, iron acquisition and metabolism, sulphur acquisition and metabolism, and nitrogen metabolism functions (as previously noted).

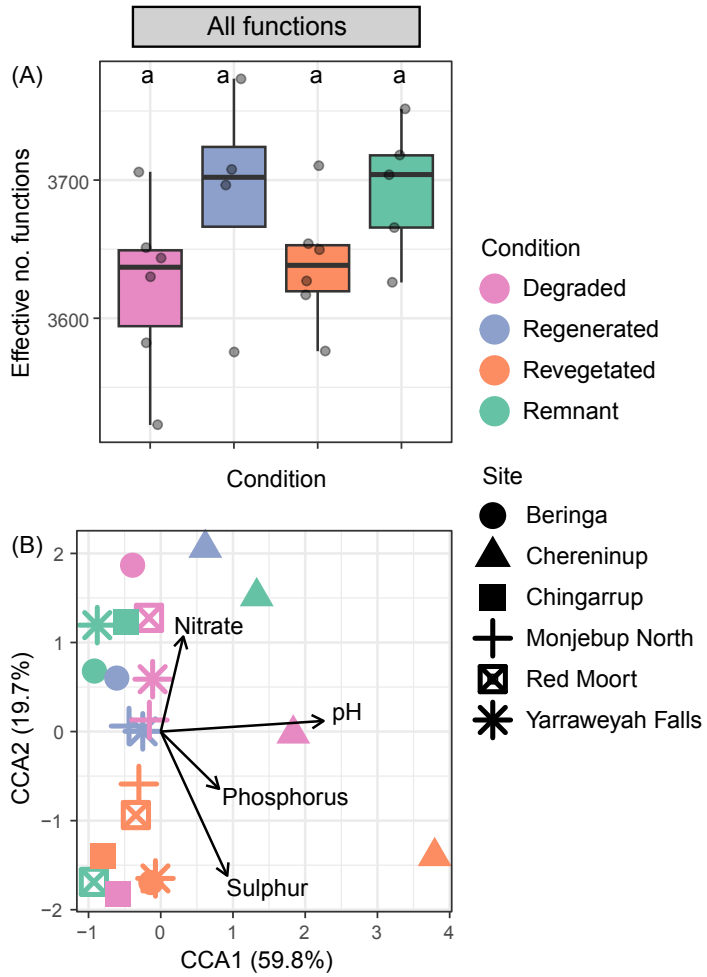


***Figure 3.*** *Microbial functional diversity, composition and associations with soil abiotic properties for all functions at the functional process level. (A) Effective number of functions (alpha diversity) in soil samples collected across four land conditions. Groups sharing the same letter do not differ. (B) Constrained correspondence analysis (CCA) indicating the composition of microbial functions in soil samples across four land conditions and the model selected soil abiotic properties that associate with those functional compositions.*

### 3.3 Phosphorus metabolism functions

When looking at functions within phosphorus metabolism, we found remnant samples had the highest effective number of phosphorus metabolism functions and was significantly greater than the effective number of phosphorus metabolism functions from degraded and revegetated land conditions (Table S2, Figure 4A;

ANOVA, *df* = 3, *F* = 7.7 *p* = 0.001). While we only found weak evidence that the composition of phosphorus metabolism differed across land condition (Table S3, Figure S1C; PERMANOVA, condition *df* = 3, *F* = 1.68, *p* = 0.079, site *df* = 5, *F* = 2.91, *p* = 0.001), functional compositions across land condition associated with phosphorus, nitrate, copper, pH, and sulphur (Figure 4B). While available (Colwell) phosphorus levels did not significantly differ across our land conditions (Figure 4C; ANOVA, *p* = 0.352, phosphorus levels were generally lower in remnant soils and 2-3 times higher in revegetated soils (remnant 3.32 $\pm$ 2.14, degraded 6.52 $\pm$ 6.73, regenerated 9.07 $\pm$ 8.11, revegetated 10.3 $\pm$ 7.63). Kendall's rank correlation test showed a moderate negative correlation between phosphorus levels and phosphorus metabolism functional diversity (Figure 4D; $\tau$ = -0.38, *z* = -2.38, *p* = 0.018).

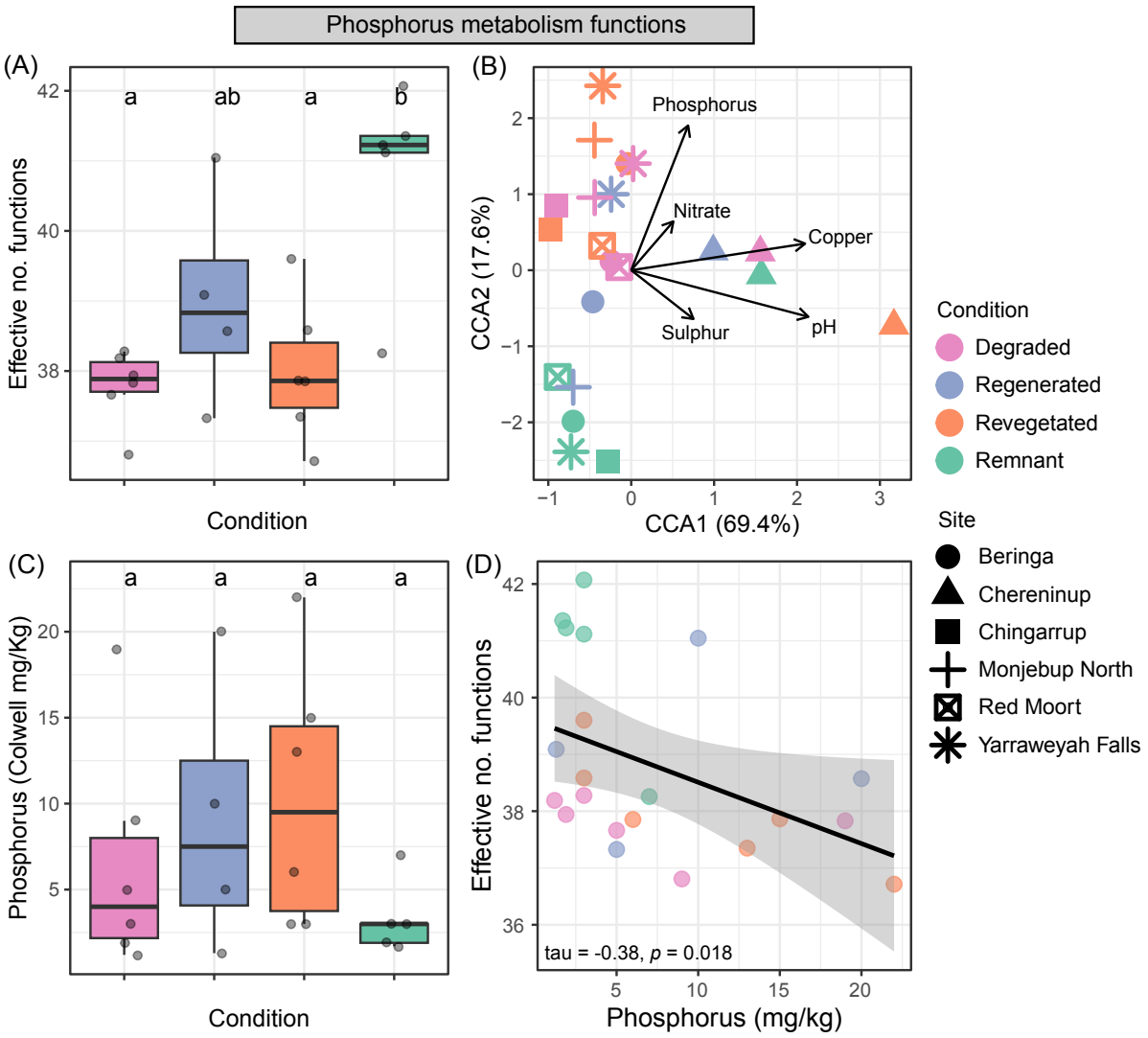


***Figure 4.*** *Functional diversity, composition and associations with soil abiotic properties for phosphorus metabolism functions at the functional process level. (A) Effective number of functions (alpha diversity) in soil samples collected across four land conditions. Groups sharing the same letter do not differ. (B) Constrained correspondence analysis (CCA) indicating the composition of phosphorus metabolism functions in soil samples across four land conditions and the model selected soil abiotic properties that associate with those functional compositions. (C) Phosphorus (Colwell) levels in soil samples collected across the four land conditions. Groups sharing a letter do not differ. (D) Correlation (Kendall's Tau) of effective number of phosphorus metabolism functions and soil phosphorus levels.*

We found no difference in the relative abundance of any specific phosphorus metabolism subsystem 3 functional group across land conditions (Figure S3; permuted ANOVA, $P > 0.05$ for all). At the function level, two phosphorus metabolism functions (Phosphonate utilization associated acetyltransferase and Ribose 1,5 bisphosphate phosphokinase PhnN EC (2.7.4.23)) had different Log Fold Changes in abundance across degraded, regenerated or revegetated land conditions compared with the remnant condition (Figure 5, Table S4).

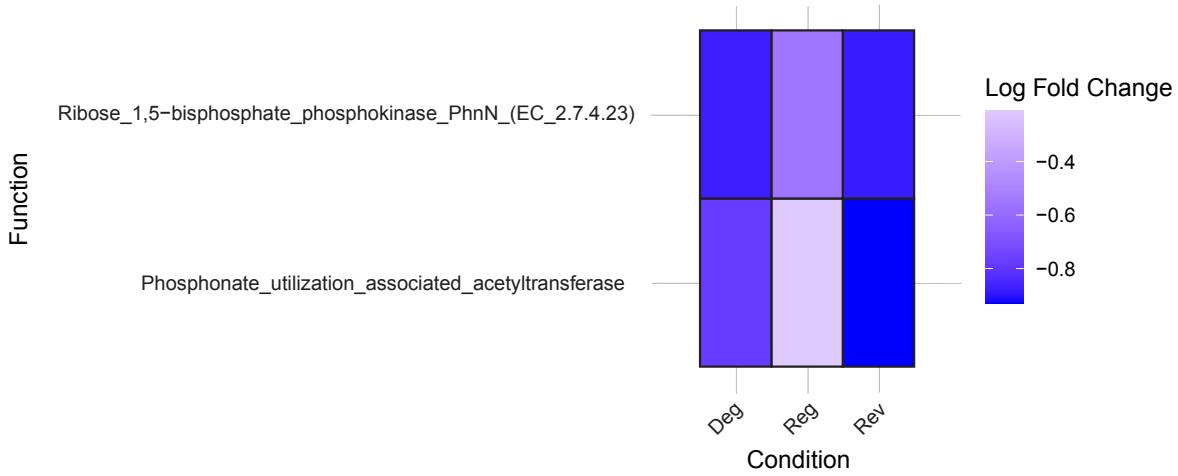


***Figure 5.*** *Heatmap of significant differential abundance (log fold change $p < 0.05$) in phosphorus metabolism functions with pairwise comparisons of degraded (Deg), regenerated (Reg) and revegetated (Rev) land conditions to the remnant condition.*

### 3.4 Other functions

For all the other subsystem 1 functional groups we assessed (i.e., amino acids and derivatives, carbohydrates, clustering-based subsystems, DNA metabolism, regulation and cell signalling, iron acquisition and metabolism, and sulphur acquisition and metabolism), only the DNA metabolism functional group had a difference in effective number of functions with remnant differing to revegetation (Figure S4; Tukey, *diff* = 3.41, 95% CI [0.23, 6.59] *adj p* = 0.03, remnant 118.31 ±

2.00, regenerated 117.68 ± 1.85, degraded 115.92 ± 2.23, revegetated 114.90 ± 1.16). The composition of functions for most subsystem groups differed across land condition (Table S3, Figure S1). Although functional compositions in all other subsystem groups associated with various soil abiotic properties, specific trends of associations across land conditions were less clear than the associations with compositions at one site in particular (i.e., Chereninup; see Figure S5). For the relative abundance of subsystem 2 or 3 groups within each of the assessed subsystem 1 groups, there were few groups that differed across land condition (Figures S6-S13).

# 4. Discussion

Here we show that despite compositional differences in soil microbial communities and their functions, overall alpha diversity of functions did not differ across actively revegetated, passively regenerated, degraded, and remnant land conditions. However, we report a negative correlation between available soil phosphorus levels and the diversity of phosphorus metabolism functions. This relationship was highlighted in remnant soils which contained the highest effective number of phosphorus metabolism functions despite having the lowest levels of available phosphorus. Furthermore, we show numerous associations between functional compositions and soil abiotic properties, in the ordination based modelling and visualisation of abiotic drivers of beta diversity – considering all functions combined, specific functional groupings (subsystems), and phosphorus metabolism. In particular, we see that abiotic properties strongly associated with the functional disparities between land conditions (i.e., separation in ordination space), which is especially evident between remnant and revegetated conditions. Together, these

results indicate that despite differences in functional compositions, aside from phosphorus metabolism functions, functional capacity as represented by the effective number of functions (overall or within subsystems) largely does not differ between remnant and the other land conditions. Although, we note that our moderate sample size may contribute to statistical similarities in the functional alpha diversities (within land condition n = 6). In any case, the fact that functional compositional differences are being maintained, especially between revegetated and remnant conditions, suggests that persistent agricultural land-use legacies remain and may indicate potential alternative stable states that are impeding complete recovery.

The higher effective number of phosphorus metabolism functions in remnant soils that have lower available phosphorus levels suggests a diversification of phosphorus acquisition strategies as a long-term microbial adaptation to phosphorus limitation. This result is further supported by the negative correlation between available phosphorus and the effective number of phosphorus functions. This may reflect a greater functional redundancy within phosphorus metabolism pathways in remnant soil, allowing microbial communities to maintain diverse mechanisms for phosphorus acquisition and cycling needed under nutrient-poor conditions (Yao *et al.*, 2018; Oliverio *et al.*, 2020). In contrast, the lower phosphorus metabolism functional diversity in revegetated and degraded soils likely indicates a reduced need for diverse phosphorus cycling pathways as microbes in high-phosphorus soils preferentially use inorganic phosphate rather than phosphonates as it requires less energy investment (Condron, Turner & Cade-Menun, 2005). While we only found weak evidence of compositional differences in phosphorus metabolism functions, these differences were strongest between remnant and revegetated conditions and

also associated with differences in soil abiotic properties, particularly with decreased phosphorus levels in remnant soil. Moreover, the repeated associations of soil phosphorus levels with the compositions (beta diversity) of multiple other functional subsystems, as well as to taxonomic beta diversity, provides further evidence for the strong role of post-agricultural phosphorus legacies impacting the recovery of microbial communities and their functions (Peddle *et al.*, 2024a; Wang *et al.*, 2024). Our findings here also support previous research that has shown phosphorus availability is a key soil property that shapes microbial and functional compositions (Delgado-Baquerizo *et al.*, 2017; Liu *et al.*, 2012; Oliverio *et al.*, 2020).

Although we did not identify any differences in the relative abundances of subsystem 3 phosphorus metabolism levels (e.g., Alkylphosphonate utilisation, P uptake cyanobacteria, high affinity phosphate transporter, control of PHO regulon etc.), two specific phosphorus metabolism genes at the functional process level (Phosphonate utilization associated acetyltransferase and Ribose 1,5 bisphosphate phosphokinase PhnN EC) involved with microbial degradation of phosphonates were less abundant in revegetated, degraded, and, to a lesser extent, regenerated soils compared to remnant soils. Agricultural soils in Western Australia have a history of extensive application of superphosphate fertiliser, which can lead to a legacy of elevated phosphorus even decades after revegetation and restoration (Lambers *et al.*, 2011; Parkhurst *et al.*, 2022b). The decreased abundance of these two genes in revegetated post-agricultural soils likely reflects a shift away from phosphorus-limited microbial strategies due to higher residual phosphorus. Together, these findings indicate that while overall phosphorus functional diversity appears highest in remnant soils, the specific metabolic pathways utilised by microbial communities differ in post-

agricultural land conditions, potentially influencing phosphorus cycling dynamics and long-term ecosystem recovery.

We show that several abiotic soil properties strongly influenced microbial community and functional compositions, with nitrate, pH, phosphorus, sulphur, aluminium, and iron each associating with either microbial community or functional compositions. These findings reinforce the importance of soil abiotic properties in shaping restoration outcomes (Robinson *et al.*, 2024b) highlighting that outcomes are not solely dictated by vegetation establishment but are also constrained by soil legacies from past land use (Parkhurst *et al.*, 2022a; Peddle *et al.*, 2024a; Toledo *et al.*, 2018). While overall functional diversity does not differ across our land conditions, subtle differences in functional composition suggests that functions in revegetated areas do not fully mirror those in remnant ecosystems. However, these subtle differences in functions do not necessarily reflect a 'lack of recovery' or dysfunction *per se*, but likely reflect altered functional dynamics resulting from alternative stable states in restoration sites with unaddressed land-use legacies (Suding *et al.*, 2004). If full functional recovery to match remnant conditions is the goal of a restoration project, addressing these constraints through targeted soil translocations/inoculations and/or biostimulation strategies (e.g., ecological phage therapy, Davies *et al.* 2024; sonic restoration, Robinson *et al.* 2024a) may be beneficial to accelerate microbial functional recovery in soil ecosystems. Moreover, further research of functional recovery using RNA-based metatranscriptomic sequencing is needed to confirm these functions are actually being expressed in the environment (Breed *et al.*, 2019). The metagenomic sequencing methods we use

here are limited in that they can only be used infer functional potential from the presence of genes, not their active expression (Cordier *et al.*, 2019).

The strong association between the diversity of phosphorus metabolism functions and available soil phosphorus levels indicates that post-agricultural abiotic legacies continue to shape microbial functions, potentially constraining long-term nutrient cycling and plant-microbe interactions. This suggests that restoration efforts should not only focus on vegetation recovery but also on strategies to promote microbial functional traits that support ecosystem processes. Targeted interventions, such as soil inoculations with microbial communities sourced from remnant ecosystems, including consideration of appropriate timing to partner with (e.g., facilitate or follow) successional establishment of host plants, may help restore pre-disturbance functional compositions. The effectiveness of such approaches will depend on whether inoculated microbiota can overcome prior residence effects and biotic resistance, persist and ideally integrate into existing communities. Together, these clear knowledge gaps emphasise the importance of research into the drivers of microbial community assembly and functional recovery in restored landscapes (Gomes *et al.*, 2025; Peddle *et al.*, 2024b).

## Acknowledgements

The authors wish to thank all landholders – Bill and Jane Thompson, Eddy and Donna Wajon, and Bush Heritage Australia – for their generosity and assistance with accessing sites used in this study. We thank Michelle Hall, Jaclyn Smith, and Alex Hams for assistance with soil sampling, field work, and other on-site and project logistics. This research was supported by funding from The Holsworth Wildlife

Research Endowment through the Ecological Society of Australia, a philanthropic donation from Peter and Maxine Wilshaw to Bush Heritage Australia, and the Australian Research Council (grant numbers LP190100051, LP240100073), and the New Zealand Ministry of Business Innovation and Employment (grant UOWX2101). We acknowledge and pay respect to the Traditional Owners of the land on which this study was conducted: the Minang and Goreng people of the Noongar Nation.

# Chapter 4:

# Stronger together: intact soil translocation increases the resilience of inoculated microbial communities

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This chapter has been prepared for submission to *Ecology Letters*. A preprint of this manuscript is available on *Authorea* at:

https://doi.org/10.22541/au.174180429.99271478/v1

Throughout this chapter I use the pronoun *'we'* to acknowledge the contributions of my coauthors, following the customary etiquette in published manuscripts.

## Abstract

Soil microbiota are fundamental ecosystem components capable of driving ecosystem recovery. However, their effective integration into ecosystem restoration efforts remains unrealised. Despite growing interest, there are limited experimental assessments on how to implement soil translocations to effectively inoculate whole microbial communities in restoration contexts. By embedding a soil translocation experiment into a restoration project in a global biodiversity hotspot, we show that retaining soil structural integrity through intact soil translocations is important in achieving successful inoculation. By contrast, surface spreading – the predominant method of soil translocation – saw microbial communities diverge away from the microbial profile of donor sites. Our findings suggest that the restoration sector should rethink its approach to microbial inoculations and consider the benefits of retaining structural integrity in translocated soils. Upscaling of investments and innovation are required to meet the increasing demand for soil translocations capable of effectively driving ecosystem recovery.

# 1. **Introduction**

Using soil microbiota directly has clear potential to improve ecosystem restoration outcomes (Coban *et al.*, 2022; Robinson *et al.*, 2023) as they are critical to ecological processes (e.g., nutrient cycling, soil formation). However, despite the recovery of soil microbiota increasingly being assessed following restoration interventions (van der Heyde *et al.*, 2022; Mohr *et al.*, 2022), soil microbiota are poorly integrated into ecosystem restoration. While post-restoration monitoring has identified patterns of soil microbiota recovery, large recovery debts can persist decades after restoration plantings (Watson *et al.*, 2022). These persistent recovery debts highlight the need to improve restoration interventions that specifically target soil microbiota to improve restoration outcomes.

Soil translocation – the movement of topsoil from a donor to a recipient site – is increasingly used as a restoration intervention to inoculate entire microbial communities or select microbial taxa into restoration sites (Dadzie *et al.*, 2024; van der Bij *et al.*, 2018). These soil translocations can be effective in driving recovery of above- (e.g., vegetation) and below-ground (e.g., microbiota) ecosystem components in some contexts (Han *et al.*, 2022; Wubs *et al.*, 2016a). However, there is a lack of research informing optimal soil translocation methods and further refinements are needed (Gerrits *et al.*, 2023; Gomes *et al.*, 2025).

The predominant soil translocation method used in restoration is surface spreading (Contos *et al.*, 2021; Gerrits *et al.*, 2023), where soil is collected from a donor site – ideally a nearby remnant site – transported to the recipient site and spread over the surface (Wubs *et al.*, 2016a; Bullock, 1998). Recipient sites are often prepared by

removing existing topsoil, but sometimes donor soil is spread directly on top of existing surface soil. Inoculation effectiveness has been shown to improve with increasing soil volume due to a higher inoculation 'dose' (Han *et al.*, 2022), however, this comes at the cost of increasing the volume of soil required from donor sites risking greater ecological impacts (Peddle *et al.*, 2024b).

Surface spreading involves the mixing of distinct soil microhabitats, along with their corresponding microbiota, resulting in a homogeneous soil environment. This convergence of distinct microhabitats and microbial communities can drive compositional changes (West & Whitman, 2022), affecting their likelihood of establishment. Microbial taxa vary in their response to disturbance of soil structure (van der Heyde *et al.*, 2017). These varied responses can impact on predictions of community-level changes during the collection, transport, homogenisation and spreading of soil in translocations. For example, disrupting soil structural integrity by mixing can reduce bacterial richness, steering communities towards more homogenous compositions and favouring faster growing, generalist taxa (West & Whitman, 2022). Therefore, preserving soil structural integrity during translocation may help retain donor communities and improve establishment of translocated microbiota, but there are no studies that assess the impact of varying soil disturbances during translocation.

As an alternative to surface spreading, intact soil translocation involves collecting intact sods, turfs or cores, and translocating these directly into the recipient restoration site (Bullock, 1998; Gerrits *et al.*, 2023). The structural arrangements of soil comprise of physical (e.g., aggregates and pores) and biological (e.g., soil

organic matter) legacies that have typically formed over decades and are key to soil functioning (Or, Keller & Schlesinger, 2021; Rillig, Muller & Lehmann, 2017). Thus, the objective of intact soil translocation is to preserve this soil structural matrix, which should result in the maintenance of the physical and biological legacies and their associated microhabitats and functions (Boyer *et al.*, 2011; Butt *et al.*, 2022). Similarly to surface spreading, studies of intact soil translocations have examined differing soil quantities and depths, usually in the 1-2 $m^2$ range and soil depths of 10-30 cm. Most intact soil translocation studies have focussed on vegetation (Cordier *et al.*, 2019; Kardol, Bezemer & Van Der Putten, 2009; Aradottir, 2012) or soil fauna (Butt *et al.*, 2022; Moradi *et al.*, 2018) community responses, with mixed results. While intact soil translocations have led to the recovery of soil microbial biomass and functional diversity (Waterhouse *et al.*, 2014), their effectiveness compared directly to surface spreading remains untested.

Given that soil microbiota are sensitive to soil structural disturbance (West & Whitman, 2022), intact soil translocations could result in improved establishment of soil microbiota compared with surface spreading. While scaling up intact translocations presents logistical challenges, intact translocation sites could serve as high-quality restoration nodes or soil biodiversity refuges. Over time, these nodes may facilitate the dispersal of beneficial soil microbiota into surrounding soils, creating a positive spillover effect. However, differences in abiotic factors such as soil pH, moisture, and nutrient levels can limit microbial dispersal from translocated soils to adjacent environments (Fierer, 2017). Despite these barriers, mechanisms such as water flow and active microbial motility can enable short-range dispersal, suggesting some level of microbial exchange is possible (Chen *et al.*, 2020; King &

Bell, 2022). While microbial dispersal from translocated soil holds promise for the wider restoration of soil biodiversity, dispersal remains largely unpredictable (Choudoir & DeAngelis, 2022).

Here, we conducted an experimental soil translocation field trial embedded in a restoration project situated within a global biodiversity hotspot in south-west, Western Australia. We compared three different soil translocation methods that aimed to isolate the effects of soil disturbance during translocation from the effects of establishment barriers at the recipient site (e.g., inoculation depth, abiotic legacies). Our treatments were (a) intact soil cores, (b) mixed soil cores and (c) surface spreading. Our first hypothesis was that reduced soil disturbance (i.e., the intact soil core treatment) would positively associate with the establishment of translocated soil microbiota due to soil microbiota being sensitive to structural disturbance and soil homogenisation alone being capable of driving divergence in microbial composition (West & Whitman, 2022). Our second hypothesis was that if we saw improved establishment of microbiota in the intact cores stemming from the reduced soil disturbance, this would result in greater dispersal of soil microbiota from the intact cores into the surrounding recipient site soil.

# 2. Materials and Methods

### 2.1 Study Site

This study was conducted across two post-agricultural restoration sites, Monjebup North Reserve and Red Moort Reserve in southwest Western Australia (Fig 1). The sites reside within the southwest Australian floristic region – a global biodiversity hotspot with exceptional levels of plant species richness, endemism, and habitat

fragmentation from land clearing (Myers *et al.*, 2000). Restoration plantings occurred in Monjebup in 2014 and Red Moort in 2015 (see Jonson (2010) and Peddle *et al.* (2024a) for further site and revegetation details). Previous soil biodiversity monitoring at these sites indicated a lack of bacterial community recovery (Peddle *et al.*, 2024a), making them ideal for testing the effectiveness of soil translocations.

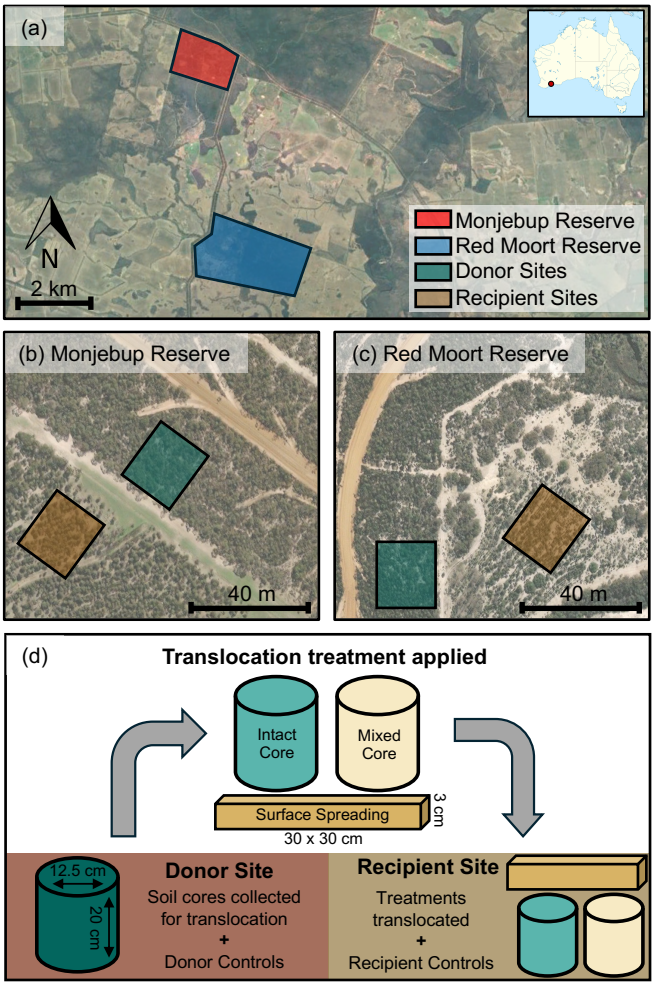


***Figure 1.*** *Map of the study locations in southwest Western Australia indicating (a) the locations of the two sites at Monjebup North Reserve and Red Moort Reserve in southwest Western Australia; the 20 m x 20 m donor plots in remnant bushland and the 20 m x 20 m recipient plots in revegetated areas at both (b) Monjebup North Reserve and (c) Red Moort Reserve. (d) graphical illustration of the experimental design showing the soil cores collected from donor sites, the experimental translocation treatments applied, and their translocation to the recipient sites.*

## 2.2 Experimental Design and T0 Sampling

Soil translocations and initial sampling (T0) occurred between 16-19 June 2022. At each site, two 20 m x 20 m plots were established; one in revegetated bushland that would receive the soil translocations (Recipient) and one in immediately adjacent uncleared remnant bushland where soil cores would be sourced for the translocations (Donor; Figure 1). Four parallel 18 m linear transects were marked out in each of the four plots. Along each transect, 18 independent experimental replicates were marked out (50 cm x 50 cm, n = 72 per site) and assigned a randomly selected translocation treatment. Along the transects in each donor plot, 54 soil cores were collected using 12.5 cm diameter x 20 cm deep stainless steel soil corers.

Soil samples (300 g) were collected from alongside every donor soil core for physicochemical and DNA analysis (detailed below). Each collected soil core then had one of three experimental translocation treatments applied: (1) Intact Core; 12.5 cm diameter x 20 cm deep soil cores kept intact during translocation; (2) Mixed Core; 12.5 cm diameter x 20 cm soil cores with the individual soil core broken-up and homogenised in a sterile plastic bag before translocation; and (3) Surface Spreading; 12.5 cm diameter x 20 cm cores that were individually homogenised identically to the mixed cores but spread in a 3 cm deep layer over a 30 cm x 30 cm area. To ensure soil translocation treatments were randomly applied to the cores collected from the donor site, we used the same randomised order from the recipient sites. Samples were also collected from three donor controls along each transect (n = 12 per site).

In the recipient plots, individual translocation treatments or recipient controls were applied to the randomly assigned independent 50 cm x 50 cm replicates along the four transects. Recipient controls did not receive any soil translocation, and a 300 g soil sample was collected from each recipient control for DNA and physicochemical analyses. For the intact core and mixed core treatment replicates, the same soil corers were used to extract a soil core which was disposed of, and donor soil from the allocated translocation treatment was placed into the resulting hole. For surface spreading replicates, surface leaf litter was removed and the homogenised soil (identical soil volume as intact and mixed cores) from the donor site was spread evenly in a 3 cm depth over the surface (30 cm x 30 cm). Plastic corflute tree guards were placed over each replicate (including the controls) to reduce the risk of interference from foraging animals. Each of the two recipient plots received a total of 14 intact cores, 14 mixed cores, 14 surface spreading, and contained 18 recipient controls. The recipient plots were also paired with 12 donor controls per site. We collected a total of 144 soil samples (300 g) across the two sites (28 intact, 28 mixed, 28 surface spreading, 36 recipient controls, and 24 donor controls). From each soil sample, 30 mL was collected in a sterile falcon tube and frozen on site until DNA extraction and sequencing. The remaining soil was sent to CSBP labs (Perth, Western Australia) for soil physicochemical analysis.

### 2.3 T1 Sampling

Soil sampling was repeated between 28-30 May 2023 (T1) to assess both microbial establishment directly in the translocated soil as well as microbial dispersal into the surrounding soil matrix. We systematically chose half of all replicates at both sites to

ensure an even resampling of the treatments and to leave enough replicates for future resampling. We also repeated sampling for the 12 donor controls in each site (i.e., n = 76 per site = 16 recipient controls, 16 intact, 16 mixed, 16 surface spreading and 12 donor controls). We collected two soil samples from each replicate: one directly from the soil translocated one year earlier to assess microbial establishment (hereafter referred to as establishment samples); and one from soil immediately surrounding the translocated soil to assess microbial dispersal (hereafter referred to as dispersal samples; n = 76 establishment, 76 dispersal).

For the establishment samples, we used a 23 mm diameter soil corer to extract 10 cm deep soil cores to collect 300 g from the intact, mixed, and both control replicates being careful to not sample surrounding soil. Due to the shallow 3 cm depth of the surface spreading replicates, a steel trowel was used to collect 300 g of soil from the top 2 cm, again avoiding any of the underlying non-translocated soil.

For the dispersal samples for intact, mixed and recipient controls, we used the 23 mm soil corers to collect 300 g of soil to a depth of 10 cm from 6 cm surrounding the translocated core avoiding any of the translocated soil. For the dispersal samples from the surface spreading replicates, we used the trowel to excavate the 3 cm layer of translocated soil and the first 3 cm of the underlying soil (to minimise contaminating the dispersal sample with translocated soil) before using the soil corer to collect 300 g of soil from under the cleared surface spreading treatment. From each 300 g sample from both establishment and dispersal samples, 30 mL was collected in a sterile falcon tube for DNA analysis and frozen on site.

## 2.4 DNA Extraction, Sequencing and Bioinformatics

For DNA extractions for both TO and T1 samples, we used the Qiagen DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and quantified concentrations fluorometrically. DNA extractions for TO samples were conducted in October 2022 and DNA was sent to the Australian Genome Research Facility (AGRF) in Melbourne, Australia for sequencing. Soil bacterial 16S rRNA from the V3-V4 region was amplified using the 341F and 806R primer set, and for fungi, the internal transcribed spacer (ITS) region in the rRNA operon was amplified using the 1F and 2R primers before 300 base pair paired end sequencing on the Illumina MiSeq platform.

DNA extractions for the T1 samples were conducted in June 2023 and DNA was again sent to AGRF for sequencing, this time using the Illumina NexSeq 2000 platform. The same gene regions (16S V3-V4 and ITS 1F-2R) were amplified using the same primer sets as the TO sequencing. T0 and T1 sequencing generated four FastQ format datasets (TO 16S, TO ITS, T1 16S, T1 ITS) for bioinformatic processing.

Due to the different sequencing platforms used across the two sample events, bioinformatics were conducted individually on each dataset using the *DADA2* pipeline (Callahan *et al.*, 2016) up to the chimera removal step. For the TO 16S and ITS datasets, FASTQ files were quality checked, and primer sequences removed using *Cutadapt* (Martin, 2011) and trimming lengths were determined for forward and reverse reads using *FIGARO* (Weinstein *et al.*, 2019). Forward and reverse reads

were trimmed, error rates inferred, paired end reads merged, and sequence tables constructed with *DADA2*.

For the T1 16S rRNA and ITS datasets, as the Illumina NextSeq platform generates binned quality scores where both *FIGARO* and *DADA2* expect non-binned quality scores, methods differ as follows: Instead of using *FIGARO* to determine trimming parameters, the forward and reverse read quality profiles were generated and visualised with *DADA2*; and at the error estimation steps, monotonicity was enforced, and loess smoothing was applied to improve error rate modelling before sequence tables were constructed. At this point the TO and T1 sequence tables were then merged (16S rRNA and ITS data handled separately) before chimera removal and taxonomic assignment. Taxonomy was assigned to the sequence tables using QIIME2 with the Silva database (v138.1) (Wang *et al.*, 2007) for 16S rRNA data and UNITE (v9.0) (Abarenkov *et al.*, 2023) for ITS data using a naïve Bayesian classifier to produce separate 16S rRNA and ITS amplicon sequence variant (ASV) abundance tables. All further statistical analyses were conducted using R (Version 4.4.0; R Core Team 2024), using the *Phyloseq* (McMurdie & Holmes, 2013) package to combine sample metadata with ASV abundance and taxonomy tables.

### 2.5 Statistics

Microbial establishment and inoculation success

We first assessed if translocated microbiota successfully established in recipient sites one-year after soil translocation and whether there were any differences in inoculation success across our three translocation treatments. Analysing only

'establishment' samples (16S rRNA and ITS), these were rarefied using the *rarefy_even_depth* function in *Phyloseq*, ensuring ASV richness was still well-represented at the chosen rarefaction levels (20,717 reads for 16S rRNA and 10,073 reads for ITS; Figs. S1, S2). Then, to assess inoculation success we used *vegdist* in *vegan* (Oksanen *et al.*, 2013) to construct a Bray-Curtis distance matrix, converted the values to a similarity (100%*(1-distance)), and plotted the similarity of each T1 treatment sample to: the mean similarity of the T1 recipient samples; and also, to the mean similarity of the T1 donor samples.

Bacterial and fungal community compositions from our three translocation treatments and two controls across both sites and sampling years were visualised with non-metric multidimensional scaling (NMDS) ordinations of Bray-Curtis distances using *ordinate* in *phyloseq*. Differences in both bacterial and fungal community compositions across translocation treatment, site and sample year were assessed with stratified permutation tests separately for bacteria and fungi (PERMANOVA) with the *strata* argument in *adonis2* to perform permutations within the levels of the specified strata (to account for each combination of site and sample year).

To assess how microbiota compositions across the different translocation treatments changed across sample years, we again used Bray-Curtis similarities comparing each sample's similarity to all donor sample similarities. The distributions of these similarity to donor values were displayed as a series of boxplots for each site/year combination for both bacteria and fungi. Kruskal-Wallis multiple comparison tests were used for each site/year combination to determine if the similarity to donor values differed across soil translocation treatments. Significant differences between

translocation treatments were then identified using post-hoc Dunn tests with Bonferroni correction to adjust *p* values for multiple comparisons.

We assessed alpha diversity by calculating the effective number of ASVs for each sample and plotting these values across translocation treatment for each site/year combination separately for bacteria and fungi. We tested the effects of soil translocation treatment, site and sample year – and their interactions – on effective number of ASVs using permuted ANOVAs with the *aovp* function in *lmPerm* (Wheeler *et al.*, 2016) separately for bacteria and fungi.

Microbial dispersal from translocated soils

Next, we assessed if translocated microbiota had dispersed into surrounding soils one year after soil translocation and whether there was any differential dispersal across our treatments. To assess if soil translocation effected microbial community compositions in surrounding soils, we excluded all 'establishment' samples from the T1 sampling event, and rarefied all remaining data based on the rarefaction curves (20,717 for 16S rRNA and 10,073 for ITS) and, following methods identically to those outlined above for microbiota establishment, assessed community-level similarities in 'dispersal' samples using NMDS ordinations and similarity to donor boxplots.

To examine potential dispersal of microbial taxa in more detail, we used differential abundance analyses at the genus level using *ancombc2* (Lin & Peddada, 2024) on unrarefied data from both establishment and dispersal samples. We ran pairwise differential analyses, comparing each soil translocation treatment – subset by either establishment samples or dispersal samples – to the recipient control samples (i.e.,

seven pairwise comparisons for each site, for both 16S rRNA and ITS). All genera with significant ($p < 0.05$) log fold changes in individual pairwise comparisons were visualised in a heatmap for each site.

Soil physicochemical changes and associations

Associations between bacterial and fungal community compositions and scaled (mean-centred and standardised) soil physicochemical variables were analysed separately for each site at T1 sampling using constrained correspondence analysis (CCA). Variables with high collinearity (>0.75) were removed and the remaining variables underwent automated model selection. Model-selected variables and their associations with bacterial and fungal composition were visualised in a CCA and tested via permutated ANOVA with 999 permutations. To explore differences in soil physicochemical variables across sampling years, each variable was compared across years within each soil translocation treatment at both sites using paired t-tests.

# 3. Results

### 3.1 Microbial establishment and inoculation success

Intact soil cores established the most donor-like communities for both bacteria (Figure 2b, Figure 3) and fungi (Figure 2c, Figure 4) at T1. At the time of soil translocation (T0), bacterial and fungal communities in donor controls and all soil translocation treatments differed to recipient controls. However, soil samples collected at T1 showed shifts in both bacterial and fungal communities, particularly the surface spreading treatment (Figure 3a, Table S1, bacteria: PERMANOVA, $p$ =

0.001 for soil treatment, site and sample year; Figure 3c, Table S2, fungi: $p < 0.001$ for soil treatment, site and sample year).

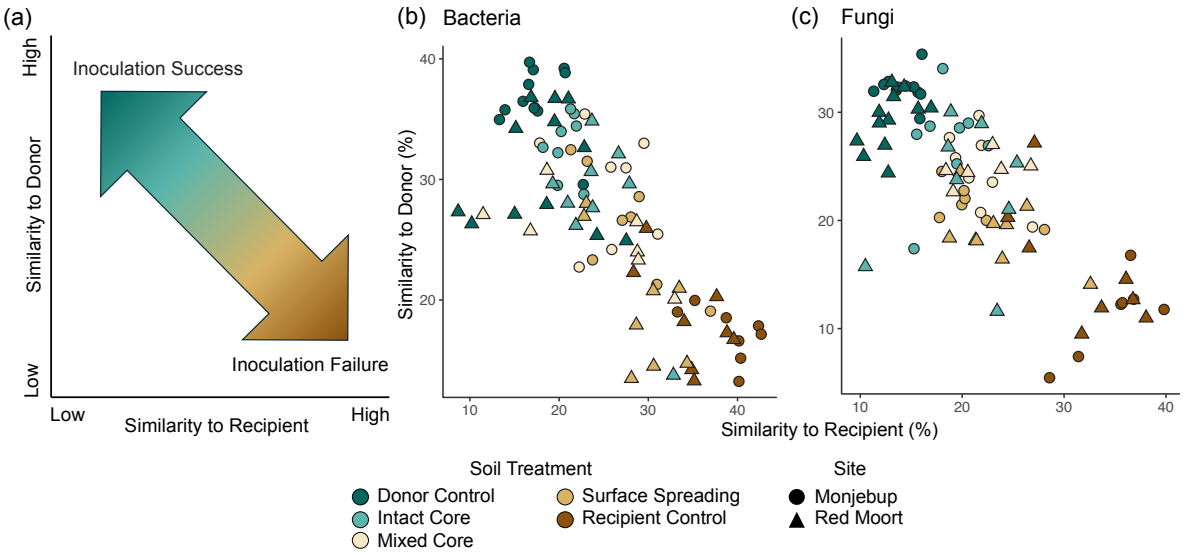


***Figure 2.*** *Success of microbial inoculations one year after soil translocation (T1). (a) Conceptual illustration to visualise establishment of microbial inoculants after soil translocations. We define inoculation success as the retention of a high similarity to donor value relative to the donor to donor similarity, whereas inoculation failure is indicated by a shift away from the donor and a high similarity to the recipient. (b) Mean similarities of bacterial communities one-year after (T1) soil translocation to both donor and recipient samples. (c) Mean similarities of fungal communities one-year after soil translocation to both donor and recipient samples.*

At T1, intact cores retained the highest similarity to donor value across both sites for both bacteria and fungi. Bacterial communities in intact cores at both sites were as similar to donors as donor control samples were to each other (Figure 3b, Table S3. In contrast, fungal communities in intact cores at both sites had lower similarity to donor values than donor controls had to each other (Figure 3d, Table S4). The mixed core treatment had the second highest community similarity to donor for bacteria at both sites and fungi at Monjebup (surface spreading had the lowest). Bacterial

communities in mixed cores at Red Moort did not differ in their similarity to donor value compared to the donor controls (Figure 3b, Table S3), although bacterial compositions at Monjebup did differ as did fungal compositions at both sites (Figure 3d, Tables S3-S4). Bacterial and fungal similarity to donor in mixed cores from both sites were still different compared to the recipient control samples.

Bacterial and fungal communities from the surface spreading treatment both diverged away from donor controls in both sites (Figures 3b 3d; Tables S3-S4). Bacterial communities in surface spreading samples at Red Moort diverged so far that their similarity to donor values were equivalent to the recipient controls but retained difference at Monjebup (Table S3). Although fungal communities in surface spreading samples at both sites had the lowest similarity to donor value of all three translocation treatments, they were still different from those in recipient controls (Table S4).

At Monjebup at T0, bacterial alpha diversity in the surface spreading, intact core and donor control samples was higher than in the recipient controls (Figure S3; Table S5). Effective number of ASVs in mixed cores at T0 did not differ to any other treatment. At Monjebup at T1, effective number of ASVs did not differ between any translocation treatment (Figure S3; Table S5). At Red Moort at T0, effective number of bacterial ASVs did not differ across translocation treatment (Figure S3; Table S5). At T1, effective number of ASVs were lower in the donor controls than the recipient controls and mixed cores (Figure S3; Table S5) but were no different than intact cores or surface spreading treatments. Surface spreading and mixed cores also differed to each other (Figure S3; Table S5). Fungal alpha diversity (effective number

of ASVs) at Monjebup at T0 did not differ across translocation treatments (Figure S4; Table S6) but was higher at T1 in intact cores than in surface spreading samples (Figure S4; Table S6). Effective number of fungal ASVs in Red Moort at both T0 and T1 did not differ across all soil translocation treatments (Figure S4; Table S6).

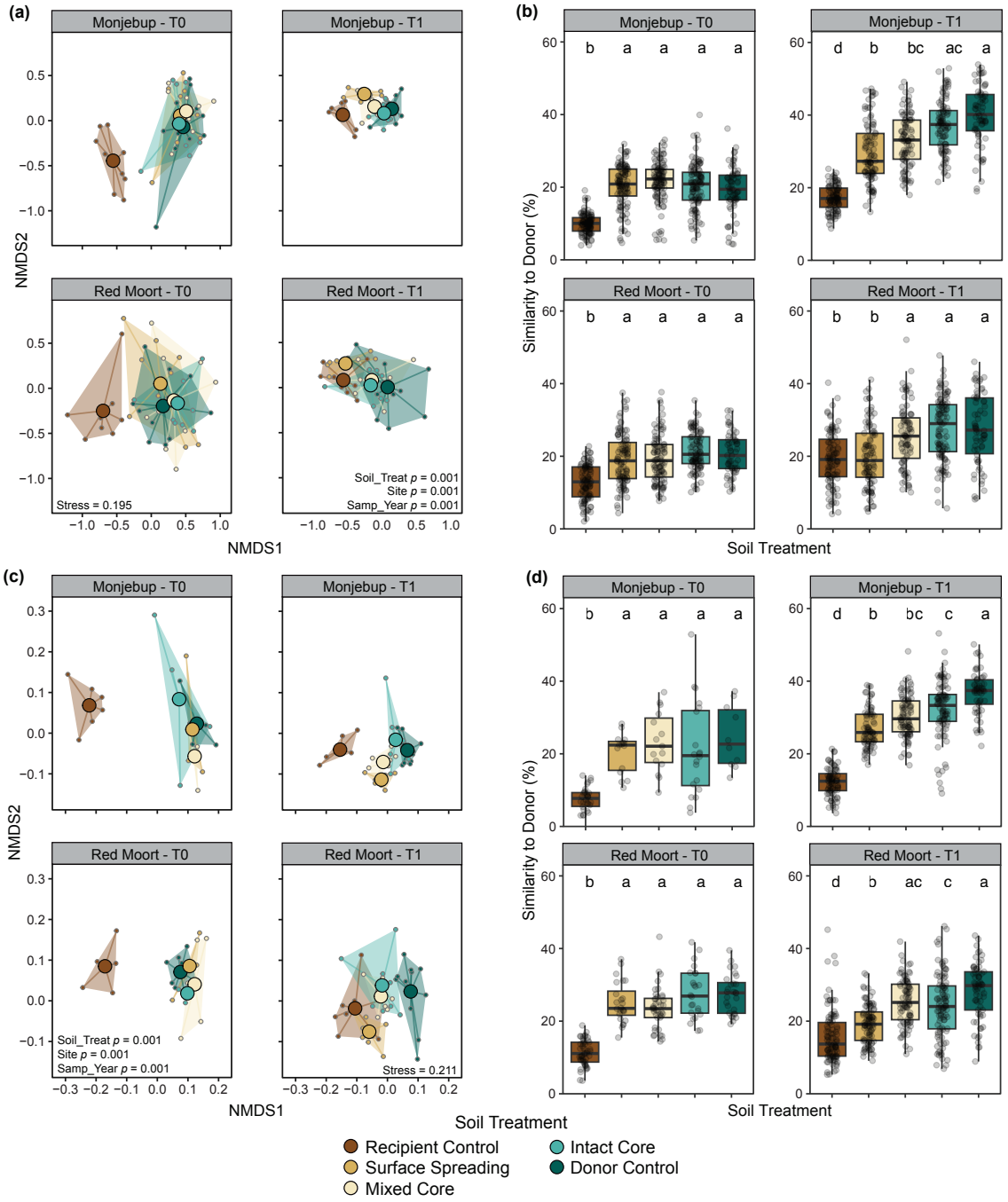

***Figure 3.*** *Community composition and similarities to Donor Controls at the time of translocation (T0) and one year post-translocation (T1). Non-metric multidimensional scaling (NMDS) ordinations for (a) bacteria and (c) fungi both faceted by site and sample year visualising changes in microbial community composition across the three translocation treatments and two controls. Statistics and stress values refer to all panels within a series. Similarity to donor boxplots for (b) bacteria and (d) fungi at both sites visualising the similarities (Bray-Curtis) of the three translocation treatments and recipient controls to the donor controls. Groups not sharing a letter are significantly different ($p < 0.05$, Kruskal-Wallis and Dunn post-hoc).*

### 3.2 Microbial dispersal from translocated soils

At the whole community level, we found no evidence that translocated soil microbiota dispersed into surrounding soil or altered soil microbial compositions at either site (Figures S5-8). At T1, bacterial and fungal mean similarity to donor values in soil surrounding the translocated cores and below the surface spreading did not differ from recipient controls but differed from donor controls (Figures S5-6; Dunn, $p < 0.05$ for donor control only). We also found no evidence at the whole community level of fungal dispersal into surrounding soils (Figures S7-8). For fungi however, mean similarity to donor values did differ between surface spreading and intact treatments at both sites (Figure S8; Monjebup surface spreading similarity to donor = 10.6 ± 3.42%, Monjebup intact similarity to donor = 13.6 ± 2.75%, Dunn $p < 0.05$; Red Moort surface spreading similarity to donor = 12.8 ± 4.44%, Red Moort intact similarity to donor = 15.3 ± 4.81%, Dunn $p < 0.05$), but all translocation treatments were similar to recipient controls and different to donor controls.

We only found evidence of differential abundances between recipient control samples and dispersal samples from each translocation treatment for a single fungal genus, *Cortinarius*, at one site (Figure 4d). This genus was higher in abundance in

the surface spreading treatment. No bacterial genus was differentially abundant between the dispersal samples from any translocation treatment and the recipient controls (Figure 4).

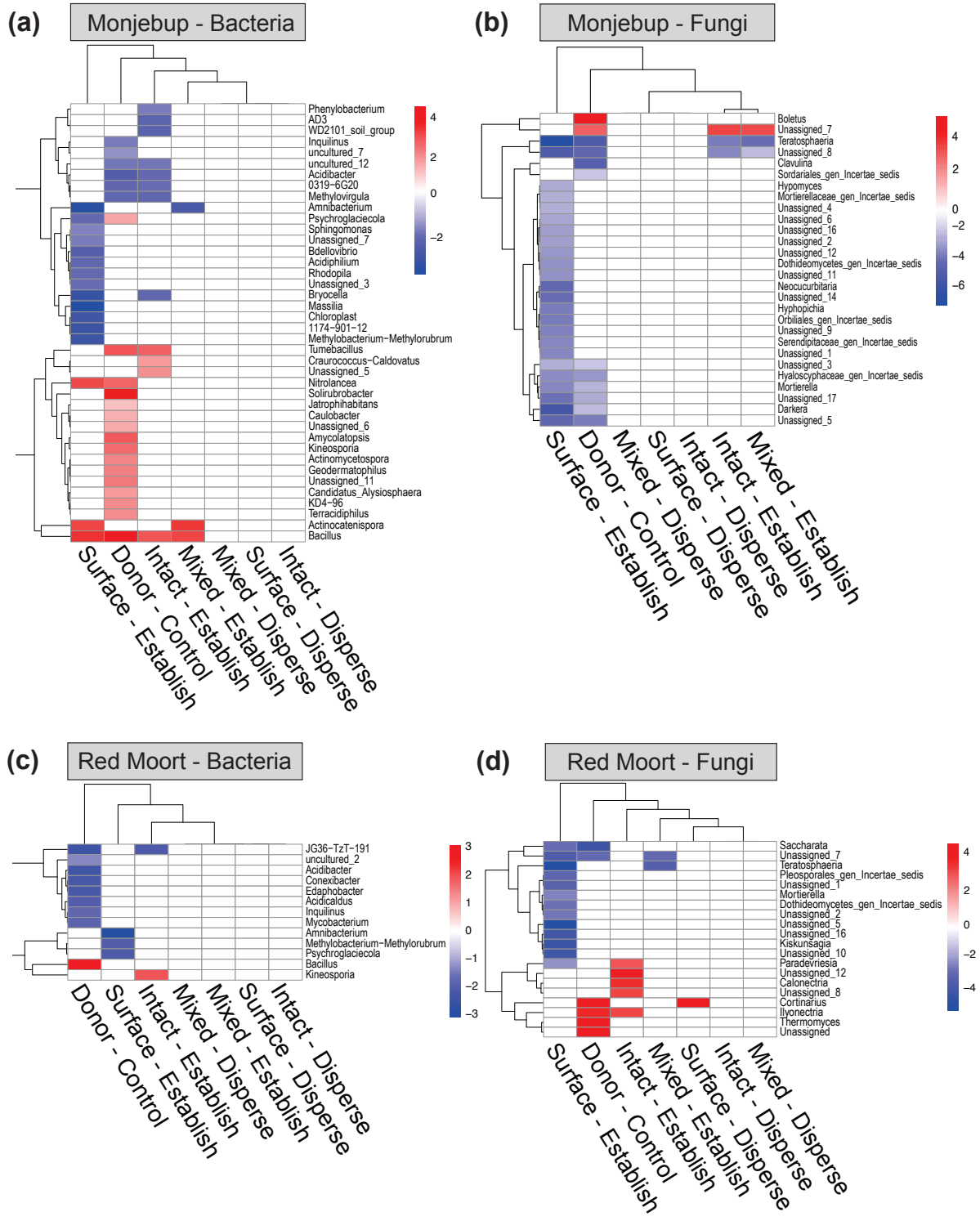

***Figure 4.*** *Heatmaps of significant differential abundance (log fold change p < 0.05) in bacterial (a, c) and fungal (b, d) genera at Monjebup Reserve (a, b) and Red Moort Reserve (c, d) assessing microbial dispersal from translocated soil into the surrounding soil. The three translocation treatment levels (Intact Cores, Mixed Cores and Surface Spreading) are split by dispersal (samples collected 6 cm away from translocated soil) and establishment (samples collected from translocated soil) levels and log fold changes across all levels including the donor control are compared to the recipient controls one year (T1) after translocation. Only a single fungal genus, Cortinarius, showed evidence of dispersal from the translocated soil into the surrounding soil and only at Red Moort.*

### 3.3 Soil physiochemical changes and associations

Bacterial communities at Monjebup associated with soil phosphorus, conductivity, sulphur and pH (Figure 5a). Increased phosphorus primarily associated with bacterial communities in recipient controls, as well as some mixed and surface spreading samples. Increased levels of pH associated with bacterial communities in mixed and surface spreading samples. Bacterial community compositions at Red Moort associated with organic carbon and pH, although patterns across specific soil treatments were less clear (Figure 5b). Fungal communities at Monjebup also associated with pH and phosphorus, as well as organic carbon (Figure 5c). Increases in both pH and phosphorus associated with fungal compositions in recipient controls as well as mixed and surface spreading samples. Fungal compositions at Red Moort associated with Sulphur and pH (Figure 5d). Although fungal communities in Donor controls largely associated with increased sulphur levels, similarly to bacterial communities at Red Moort, patterns across specific treatments were less clear than they were at Monjebup.

We found more differences in soil abiotic properties across sample years (i.e., T0 vs T1) in both mixed and surface spreading treatments than we did in either control or the intact treatment (Figures S9-10).

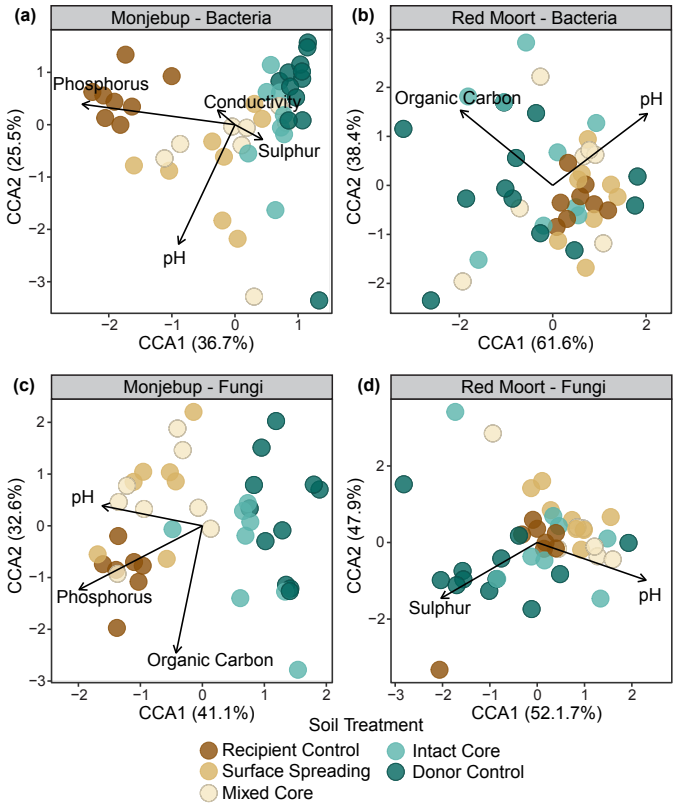


***Figure 5.*** *Constrained correspondence analysis (CCA) plots indicating associations between model-selected soil physicochemical properties and bacterial (a, b) and fungal (c, d) community compositions at Monjebup (a, c) and Red Moort (b, d).*

# 4. Discussion

We experimentally tested the effect of three soil translocation methods – intact cores, mixed cores and surface spreading – on inoculating desirable soil microbial communities in a restoration project within a global biodiversity hotspot. After one year under field conditions, microbiota translocated via intact soil cores established most effectively, with bacterial communities in particular retaining similarity to donor

controls. In contrast, surface spreading – the most common soil translocation method used in restoration – resulted in microbial communities that diverged away from donor sites, becoming more like those in recipient sites. Our study highlights the importance of preserving soil structure and microhabitats during translocation to affect successful microbial inoculations. We recommend that the restoration sector prioritises research and investment into scalable soil translocation techniques that preserve soil structure to enhance ecosystem recovery outcomes.

*4.1 Soil Structural Integrity Improves Inoculation*

We show that retaining soil structural integrity during soil translocation led to the establishment of whole microbial communities, supporting our first hypothesis. Our intact soil core treatment maintained the most donor-like bacterial and fungal compositions one year after translocation. While microbial communities in our mixed treatment did not diverge as far as those in the surface spreading treatment, they were generally less similar to donor controls than the intact treatment. This improved establishment of microbiota in intact cores likely reflects reduced disturbance during soil translocation. The difference between mixed and intact treatments in isolation underscores the impact of soil homogenisation on microbial communities. Our findings offer field-based evidence that homogenising heterogeneous soil microhabitats alters microbial communities and impacts inoculation capacity. Previous studies have shown that frequent soil mixing in microcosms increasingly diverges bacterial communities from unmixed controls (West & Whitman, 2022), underscoring how soil disturbance can affect the establishment of inoculated microbiota.

Fungal communities in our intact treatments diverged further from donor controls than bacterial communities. Fungi in natural soil systems rarely rely on sporulation and consist of extensive mycelia (Schnoor *et al.*, 2011). These contrasting life history strategies in fungi likely explain the divergence from the donor soil composition observed in the intact translocation, as even intact core extractions will disrupt fungal organisms that are reliant on extended networks of mycelia.

We show that surface spreading was not effective in establishing donor microbial communities in the recipient plots after just one year. These results were likely driven by soil homogenisation (i.e., mixing many microhabitats and their constituent microbiota) and elevated exposure to environmental influences (e.g., due to surface spreading having a high surface area). Surface spreading is the predominant soil translocation method used in the restoration sector (Contos *et al.*, 2021; Gerrits *et al.*, 2023) and although surface spreading has previously been shown to be effective in inoculating some microbiota, our results support the finding that success is often site and context dependant (Gerrits *et al.*, 2023). While our soil inoculation ‘dose’ is comparable to that used in other studies (Han *et al.*, 2022; Wubs *et al.*, 2016a), surface spreading inoculations may be more effective on loamy soils (Gerrits *et al.*, 2023) compared to the sandy soils in our study.

The homogenised soils in both mixed and surface spreading treatments appeared to be more susceptible to the soil abiotic legacies in the recipient site than the intact treatment. While we anticipated associations between soil microbiota and abiotic properties between our two controls, the associations between soil abiotic properties and the surface spreading and mixed treatments after a single year were surprising.

These associations may indicate elevated susceptibility of the translocated soils in these treatments to the abiotic legacies present in the surrounding soil at recipient sites. The features of pore space in soil (e.g., size, distribution, connectivity) are important for the biochemical processes of soil. Porosity, and the extent to which pores are saturated and connected, can affect abiotic and biotic conditions in soil (Roger-Estrade *et al.*, 2010; Six *et al.*, 2004). Here we found that the loss of physical structure in homogenised soils made them more susceptible to changes in abiotic properties. While there is strong evidence that abiotic properties and microbiota affect soil structure and aggregate formation (Or *et al.*, 2021; Rillig *et al.*, 2017; Rillig & Mummey, 2006), further research is needed to improve our understanding of how disturbance to soil structure affects abiotic and biotic properties in soil and what this means for inoculation success across varied sites and contexts.

*4.2 No Evidence of Microbial Dispersal from Translocations*

We found no evidence to support our second hypothesis as none of our three translocation methods led to the dispersal of inoculated microbiota into the surrounding soil after one year. Successful dispersal of inoculated microbiota into surrounding soils is central to the ‘restoration island’ concept (Hulvey *et al.*, 2017), where soil cores act as nodes of healthy soil biodiversity, cumulatively and positively affecting surrounding soil. While the lack of observed dispersal could simply be due to the short one-year period between re-sampling, both environmental filtering driven by the persistent agricultural land-use legacies in our sites (Peddle *et al.*, 2024a) and limited dispersal capabilities of microbes in soil are likely barriers to dispersal (Liu & Salles, 2024; Walters *et al.*, 2022; Chen *et al.*, 2020). Overcoming these land-use legacies is a major challenge facing restoration in nutrient-limited ancient soils, like

those in southwest Western Australia (Parkhurst *et al.*, 2022b; Standish *et al.*, 2006). Restoration interventions like soil scraping and removal to address abiotic legacies are costly (Gibson-Roy, Delpratt & Moore, 2024), but may be warranted to facilitate successful inoculation. Furthermore, the relatively small soil volumes in our experiment may need to be increased across treatments to increase the propagule pressure needed for microbiota establishment and dispersal into surrounding soil. Further research with increased soil volumes will be beneficial to assess if intact soil translocations still outperform surface spreading. Longer term research might also investigate repeated surface spreading inoculation episodes at intervals that allow progressive development of a range of suitable microhabitats in recipient soils, to favour diverse requirements of the donor microbiota.

While our results indicate that intact soil translocation was the most effective method at inoculating soil microbiota, scaling up intact soil translocations to effect positive restoration outcomes faces numerous challenges. Sourcing soil for translocation impacts donor sites and projects need to carefully balance the benefits of soil translocation with the impacts to remnant ecosystems. Projects with existing remnant habitat already slated for clearing (e.g., surface strip mining) would be good candidates to consider large scale intact soil translocation. Additionally, restoration sites with abiotic soil legacies that differ strongly from restoration target conditions should reassess expectations from using surface spreading translocations. Strong physicochemical differences will present a barrier to establishment and dispersal of donor microbiota. Achieving positive outcomes in such situations may require extensive action to address the physicochemical limitation, and in extreme cases soil

removal and replacement in a manner that maintains soil structure during translocation.

Overall, our findings show that maintaining soil structural integrity via intact soil translocation is important to successfully establish whole soil microbial communities. In contrast, we show that surface spreading – a widely used method of inoculating soil microbiota in the restoration sector – was unsuccessful in establishing microbial communities in the recipient site after only one year. These results highlight the impact of soil homogenisation during translocation on the establishment of inoculated microbial communities. Furthermore, our findings suggest a need for the restoration sector to reconsider soil translocation approaches and invest in scalable applications that maintain the structural integrity of soil during translocation.

## Acknowledgements

We thank Luisa Ducki, Kelsie Lambert, Emerson Lamond, Keely O'Connor, Daniel Shepherd, Nikki Maher, and Hannah Hall for their help with fieldwork, and Alex Hams and Lewi Marr for their help with on-site logistics. This research was supported by funding from The Holsworth Wildlife Research Endowment through the Ecological Society of Australia, a philanthropic donation from Peter and Maxine Wilshaw to Bush Heritage Australia, and the Australian Research Council (grant numbers LP190100051, LP240100073), and the New Zealand Ministry of Business Innovation and Employment (grant UOWX2101).

# General Discussion

## 1. Thesis synthesis

Refining how the soil microbiome is integrated into ecosystem restoration has great potential to improve restoration outcomes. However, numerous knowledge gaps and technical barriers impede this integration, and advancements are needed to progress effective restoration at the scales required to reverse the global biodiversity and climate crises (Peddle *et al.*, 2024b; Robinson *et al.*, 2023). In this thesis, I aimed to identify and address knowledge gaps that are hindering the effective integration of the soil microbiome into ecosystem restoration. Through synthesising current and emerging practical applications of the soil microbiome in restoration planning, interventions, and monitoring, in my first chapter I identify key knowledge gaps and highlight how addressing these gaps can improve restoration practices (e.g., effective soil inoculation methods) and ecological outcomes (e.g., priming microbiota to provide a fitness benefit for future plant generations).

In my second and third chapters, I use two different meta-omic techniques – amplicon sequencing and shotgun metagenomics – to assess the recovery of microbial communities and their functional capacity following post-agricultural restoration plantings in a global biodiversity hotspot. In contrast to a majority of similar studies (see Watson et al 2022), I find in chapter two that microbial communities in these sites show no clear indications of recovery and that post-agricultural abiotic soil legacies (e.g., elevated levels of available phosphorus in revegetated post-agricultural sites) are likely barriers to recovery. In chapter three, I show that despite the lack of recovery in community and functional composition, soil bacterial functional diversity – a key indicator of functional capacity – does not differ

between remnant and revegetated sites. Nonetheless, associations between post-agricultural soil legacies (e.g., available phosphorus) and altered functional compositions indicated the soil microbiome was in an alternative stable state that likely requires additional interventions (e.g., soil translocations) that directly target its recovery. Finally, in chapter four, I conducted a soil translocation experiment aimed at addressing the lack of experimental evidence on the effectiveness of different soil translocation methods for inoculating whole microbial communities in restoration sites. In this chapter, I show that maintaining soil structural integrity (via translocating intact soil cores) leads to the establishment of donor site microbial communities into recipient sites, whereas surface spreading translocations – the most common soil inoculation method – resulted in microbial communities diverging away from the donor, becoming more like communities in the recipient site.

Together, these thesis chapters provide important context for the role of the soil microbiome in ecosystem restoration. They highlight that, despite global trends (Watson et al 2022), the recovery of soil microbial communities following restoration plantings is not always the case. Further, they emphasise that the assumption that restoration plantings alone result in broader ecosystem recovery (the fields of dreams hypothesis) does not always hold true. Additionally, the strong emphasis on restoration plantings in ecosystem restoration misses an important opportunity to address whole-of-ecosystem recovery, including biodiversity in the soil. The findings also highlight that post-agricultural soil legacies are important barriers to the recovery of the soil microbiome. These legacies can lead to alternative stable states in the soil microbiome that resist recovery despite revegetation efforts. Finally, the findings highlight the potential of using intact soil translocations as a novel but

effective method of inoculating whole microbial communities into degraded ecosystems – this approach was effective at establishing a target soil microbial community over a 1 year period, despite the post-agricultural soil legacies. However, scaling up intact soil translocations to a level required to effect landscape-scale restoration presents challenges as harvesting soil comes at an ecological cost.

# 2. Harnessing the Soil Microbiome to Improve Ecosystem Restoration

In the following sections, I discuss the implications of my research findings for both restoration science and practice and outline future research directions aimed at continuing to advance restoration outcomes by better integrating the soil microbiome.

## 2.1 Improving causal understanding

Improving our causal understanding of how specific restoration efforts (e.g., restoration plantings, soil translocations) may or may not lead to the recovery of broader ecosystem components including the soil microbiome is essential to progressing ecosystem restoration (Peddle *et al.*, 2024b; Rillig *et al.*, 2024). In chapter one, I highlight numerous knowledge gaps that need to be addressed (see chapter 1, section V) to improve how the soil microbiome can be integrated into ecosystem restoration. I emphasise the need to improve our causal understanding of microbial responses to common restoration interventions that do not directly target the microbiome (e.g., restoration plantings), and the need to determine when the inclusion of microbiome-targeted interventions (e.g., soil inoculation) is worthwhile given the increase in resources required for such interventions. For example, with an

increased understanding of specific barriers to microbial recovery (e.g., agricultural land-use legacies), restoration projects will be better positioned to employ decision support frameworks to determine if minimal interventions (e.g., restoration plantings) alone will be sufficient to recover complete ecosystems, or if the investment in further microbiome-specific interventions is warranted.

Chapters two and three progress our understanding of how the most commonly deployed restoration intervention – restoration plantings of native plant communities – affects the compositional and functional recovery of the soil microbiome. Despite finding that restoration plantings in my sites were not associated with soil microbiome recovery, my work highlights that post-agricultural soil abiotic legacies can be a major barrier to recovery. This suggests that restoration practitioners should invest effort during the planning phase of restoration projects to characterise physical and chemical soil properties, as these may be effective indicators of the potential of soil microbial community recovery (Osburn *et al.*, 2021; Turley *et al.*, 2020). Furthermore, these abiotic legacies should be targeted during the early stages of restoration efforts to establish conditions that are conducive to the establishment of the target ecosystem. However, my findings here are only directly relevant in the particular ecosystem context that my studies were done, and future work needs to continue to build evidence across other ecosystem contexts. Additionally, future research should move beyond the focus on observational chronosequence based studies and embed soil microbiome recovery experiments into restoration projects (Gellie *et al.*, 2018; Broadhurst *et al.*, 2023). For further detail of future research needs specific to my study system in southwest Western Australia, see Box 1.

**Box 1** Southwest Western Australia faces unique restoration challenges that may not be faced in other ecosystems (e.g., high levels of endemism, soil-borne plant pathogens, nutrient-poor soils) (Monks *et al.*, 2019; Standish & Hobbs, 2010). As such, I present here some unresolved research questions that could be used to guide future research directions directly relevant to the sites and ecosystems studied in this thesis:

- How can persistent agricultural land-use legacies be addressed to facilitate improved restoration outcomes?
- Does further longer-term monitoring indicate any change in abiotic legacies or compositional shifts in microbial communities or functions?
- Does the preferential establishment of microbiota translocated in intact cores over mixed and surface spread treatments hold over longer timeframes?
- Can soil translocations also provide a benefit to the germination and establishment of recalcitrant and/or threatened plant species (Figure 1)?
- Can soil microbiota production areas be developed as a donor source of soil transplants with locally desirable microbial communities?
- Can intact soil core translocations be scaled up to act as *'restoration islands'* and provide a positive spillover effect to surrounding soil?

Researchers and practitioners in southwest Western Australia could establish robustly-designed experimental plots embedding different restoration treatments (e.g., common garden experiments, soil scrapping, soil translocation, seed pelleting or biopriming, nutrient amendments) into existing and new restoration sites (Figure

1). These experiments could provide further direct evidence to help improve our causal understanding of ecosystem-level responses to restoration practices.

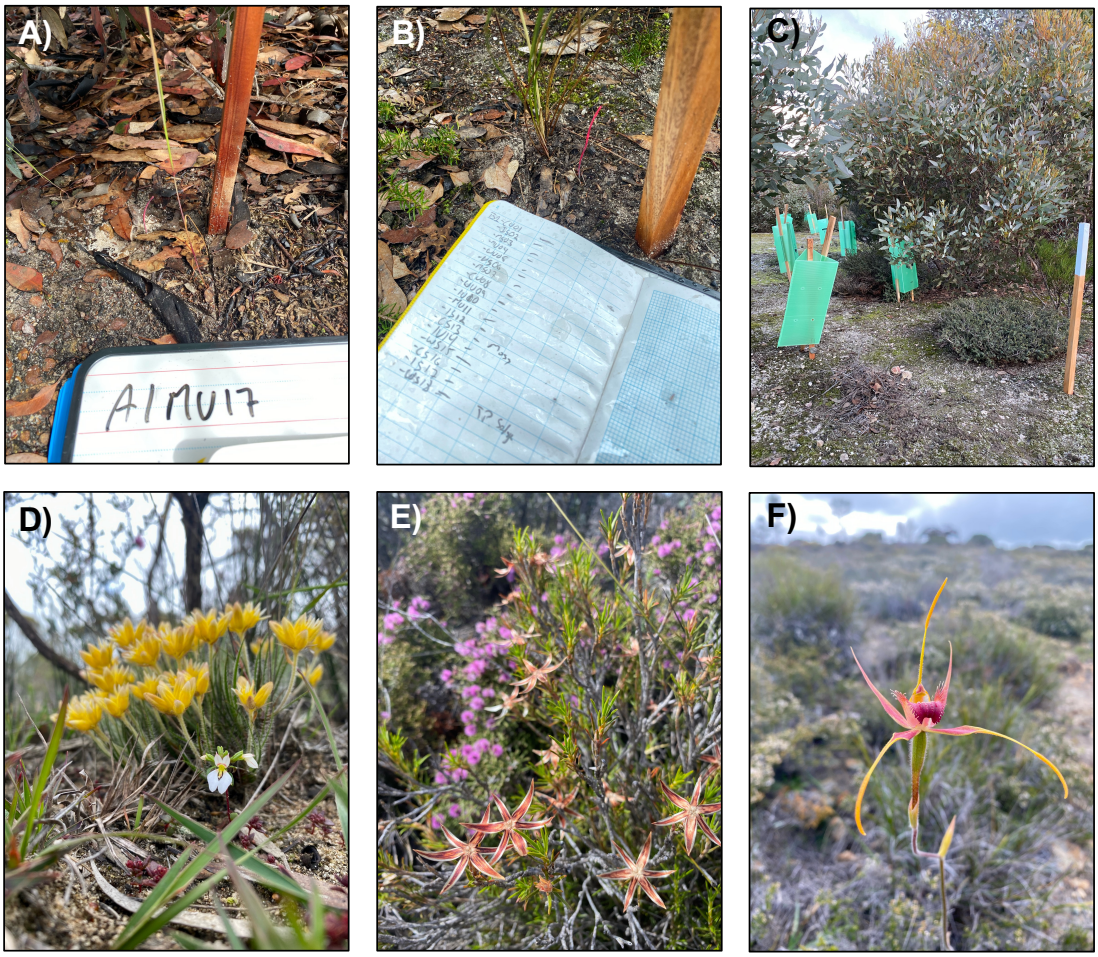


***Figure 1*** *Photos from field sites in the Fitz-Stirling region of southwest Western Australia illustrating A-B) emergence of unknown sedge species from intact soil cores 5 months post translocation; C) replicates of a soil translocation experiment embedded into a restoration site; D-F) examples of the highly diverse recalcitrant ground cover vegetation layer found in remnant sites but not post-agricultural sites.*

## 2.2 Overcoming land-use legacies

Bacterial community composition is often strongly associated with environmental factors, particularly soil physicochemical properties including pH, organic carbon, and soil nutrients such as phosphorus (Fierer, 2017; Oliverio *et al.*, 2020). This

aligns with a contentious hypothesis of microbial biogeography that *"everything is everywhere, but the environment selects"* (Baas-Becking, 1934), which posits that regardless of the potential for widespread microbial dispersal, microbial establishment and composition is largely governed by environmental filtering. Across chapters two and three, microbial taxonomic and functional composition correlated with soil abiotic properties, supporting the role that environmental conditions have on shaping microbial communities (Delgado-Baquerizo & Eldridge, 2019; Fierer & Jackson, 2006). While my results indicate environmental filters are structuring microbial communities, my research did not explicitly separate the relative contributions of different assembly processes such as dispersal limitation, priority effects, or stochastic drift. Thus, while the observed patterns are consistent with environmental selection, other ecological factors or microbial assembly processes likely also contribute to microbial compositions across my studied ecosystems. Further assessments including analyses that apply neutral community models to determine whether observed community distributions fit predictions of stochastic or deterministic processes, or reciprocal soil transplants between revegetated, degraded, and remnant sites would both be useful to provide more concrete conclusions on the relative contributions of different assembly processes.

The limited recovery of soil bacterial communities and their functions in post-agricultural landscapes despite revegetation efforts underscores the need for restoration strategies that target land-use legacies in order to improve long-term ecological outcomes. In historically phosphorus-deficient landscapes such as southwest Western Australia, both above- (e.g., vegetation) and below-ground (e.g., soil microbiota) biota have developed unique strategies to access phosphorus

(Lambers *et al.*, 2013). In restoration settings, these phosphorus legacies can impede the establishment of native plants (Daws *et al.*, 2015; Parkhurst *et al.*, 2022b) and my findings indicate this also applies to soil bacterial communities. Current restoration strategies aimed at addressing soil nutrient legacies are often insufficient (Parkhurst *et al.*, 2022b). For example, phytoremediation via temporarily continuing crop harvesting (without additional fertiliser application) to deplete nutrient levels has been shown to only moderately reduce levels of soil phosphorus (Schelfhout *et al.*, 2015). Also, while topsoil scrapping and removal off-site can be effective (Gibson-Roy *et al.*, 2024; Guevara-Torres, Zakrzewski & Facelli, 2024), it requires a suitable area to dispose of the soil and, depending on topsoil depth, may require the replacement of removed soil with new topsoil (e.g., when topsoil scrapping removes the entire layer of topsoil). Therefore, new approaches to address soil abiotic legacies are needed. Engineering microbial communities through biostimulation, microbial inoculation, or fungal-based approaches has potential to reduce excess soil nutrients (Philippot *et al.*, 2023). Potential strategies include promoting microbial communities for nitrogen immobilisation or denitrification (Gannett *et al.*, 2024) or introducing phosphorus-solubilising or denitrifying bacteria (Iftikhar *et al.*, 2024). However, numerous challenges including long-term stability and scalability remain, making this an emerging but underexplored area in restoration science warranting further research investment.

### 2.3 Function in an alternative stable state

A deeper understanding of microbial functional responses and adaptations to post-agricultural soil conditions is required to refine restoration approaches that aim to recover resilient ecosystems. Despite the general lack of differences in overall

functional capacity in my sites, the associations between functional compositions and altered soil abiotic properties indicate that land-use legacies have resulted in an alternative stable state (Suding *et al.*, 2004), which has not been overcome by revegetation alone. This finding aligns with other studies demonstrating that microbial community and functional compositions in restored sites can remain distinct to remnant sites despite vegetation recovery (Mason *et al.*, 2023; Singh *et al.*, 2024), particularly when soil conditions retain legacy effects from prior land use (Osburn *et al.*, 2021). The persistence of these functional differences highlights the need for further investigation into the ecological thresholds that govern microbiome responses to restoration. Future research should assess whether targeted interventions, such as soil inoculations or amendments (e.g., carbon additions) that alter soil abiotic properties, can initiate a shift in these stable states and facilitate microbial functional reassembly. Additionally, long-term monitoring of microbial functions, rather than cross-sectional snapshots, is needed to determine whether functional convergence with remnant ecosystems is possible over extended restoration timescales.

Despite the compositional differences of functions across restored and remnant sites, I found little evidence of a reduction in functional capacity. This may indicate that functional redundancy across the microbiome can still maintain functional capacity and allow for compositional recovery over time as environmental conditions shift in response to land-use change and restoration actions (Amarasinghe *et al.*, 2024). However, further longitudinal research in these sites would be needed to determine if natural succession alone is capable of triggering regime shifts away from the current stable state (Ranheim Sveen, Hannula & Bahram, 2024).

Experiments that manipulate the associated environmental condition (e.g., available phosphorus) would also be useful to determine if functional compositions recover with a recovery in environmental conditions and inform specific management actions. Importantly though, shotgun metagenomics faces limitations in that it is only capable of quantifying the presence of functional genes in a sample and not their actual active expression in an environment (Sun & Ge, 2023). As such, further research using RNA based sequencing approaches like metatranscriptomics to assess differences in active gene expression would be beneficial to elucidate further functional insights and confirm functional capacities. Regardless, the lack of recovery in taxonomic and functional compositions as well as soil abiotic properties warrants further research into the efficacy of microbiome-focussed restoration interventions (e.g., soil translocation and microbial inoculation) for directly restoring the soil microbiome.

### 2.4 Scaling-up microbial-focussed restoration strategies

Achieving large-scale ecosystem restoration will require an upscaling of innovation and investment to ensure that soil microbiome-focused restoration strategies are both ecologically effective and logistically feasible. Despite research showing positive overall effects of soil translocations for restoration (Han *et al.*, 2022; Wubs *et al.*, 2019a), there is still a deficiency of evidence regarding the most effective methods of inoculating whole microbial communities (Gerrits *et al.*, 2023; Gomes *et al.*, 2025). While my fourth chapter found that the novel use of intact soil translocations resulted in improved establishment compared to the commonly-used surface spread inoculation, scaling up intact soil translocations will be challenging. One approach is based on the ‘restoration island’ concept where more intensive and

costly restoration approaches are limited to nodes of the highest quality restoration with the expectation that benefits will spillover to surrounding areas over time (Hulvey *et al.*, 2017). However, while my results indicated successful establishment, I found no evidence to support microbial spillover from translocated soils into the surrounding soil. As the relatively short one-year resampling period in my study may be insufficient time to observe any spillover effects, further longitudinal research is needed to determine if this result stands over longer timeframes. Additionally, future studies should also assess increased soil volumes or core sizes. Because the effect of inoculation can increase with greater soil volumes (Han *et al.*, 2022), translocating larger cores may result in an increased chance for dispersal and greater positive spillover effects on the surrounding soil matrix.

Upscaling intact translocations to entirely cover large restoration sites faces numerous challenges, primarily regarding access to suitable sources and volumes of donor soil. Determining cost effective techniques to extract larger volumes of soil while limiting disturbance to soil structural integrity will also be challenging. Research aimed at addressing both of these challenges could leverage collaborations with organisations with the requisite mechanical, logistical and financial resources. Opencast mining operations – such as those in Western Australia's Jarrah Forest – already use heavy equipment to collect topsoil from soon-to-be mined areas and transport it to previously mined areas as a part of their rehabilitation practices (Tibbett, 2010). Given their pre-existing investment in large-scale soil translocations and the links between improved restoration outcomes and social licence (Tibbett, 2024), mining companies would be in an ideal position to embed upscaled intact soil translocation experiments to address these continued knowledge gaps. Outside of

situations where restoration projects have access to sufficient and suitable remnant soil reserves, the idea of soil microbiota production zones – similar to seed production zones (Zinnen *et al.*, 2021) – warrants further research. However, the concept of soil microbiota production areas is still undeveloped and relies on our ability to identify appropriate communities and curate them efficiently and at scale. Furthermore, as large scale soil translocations have the potential to degrade the very remnant ecosystems we are trying to recreate, robust risk analyses and decision-support frameworks need to be developed to determine when and where these restoration interventions will be justified.

## 3. Conclusion

Effective ecosystem restoration must extend beyond restoration plantings alone and explicitly consider soil microbial communities and their functional roles in ecosystem processes. This thesis advances both theoretical and applied aspects of restoration ecology by demonstrating that post-agricultural legacies can create alternative stable states in soil microbiomes, potentially constraining microbial recovery despite successful revegetation. My findings highlight that common restoration interventions, such as restoration plantings, should not be relied upon to cause microbial community or functional convergence with remnant ecosystems. Instead, persistent soil abiotic legacies, particularly altered nutrient profiles, shape microbial functional compositions in ways that resist natural reassembly. Importantly, my research provides experimental evidence that soil translocation methods – especially intact soil cores – can facilitate microbial establishment in degraded landscapes. However, upscaling such interventions remains a major challenge, requiring innovative approaches to balance ecological benefits with logistical feasibility. Future research

must focus on optimising microbiome-targeted restoration strategies, including soil inoculations, microbial bioengineering, soil microbiota production areas, and integrated restoration frameworks that account for microbial (re)assembly processes. Additionally, long-term studies are needed to assess whether microbiome recovery trajectories eventually align with broader ecosystem restoration goals. By bridging microbial and restoration ecology, this work reinforces the necessity of explicitly considering microbial communities in restoration planning, interventions and monitoring, ensuring that future efforts move beyond aboveground-focused paradigms toward holistic, functionally resilient ecosystem recovery.

# References


Abarenkov, K., Nilsson, R. H., Larsson, K.-H., Taylor, Andy F. S., May, Tom W., Frøslev, T. G., Pawlowska, J., Lindahl, B., Põldmaa, K., Truong, C., Vu, D., Hosoya, T., Niskanen, T., Piirmann, T., Ivanov, F., Zirk, A., Peterson, M., Cheeke, Tanya E., Ishigami, Y., Jansson, Arnold T., Jeppesen, Thomas S., Kristiansson, E., Mikryukov, V., Miller, Joseph T., Oono, R., Ossandon, Francisco J., Paupério, J., Saar, I., Schigel, D., Suija, A., Tedersoo, L. & Kõljalg, U. (2023). The UNITE database for molecular identification and taxonomic communication of fungi and other eukaryotes: sequences, taxa and classifications reconsidered. *Nucleic Acids Research* **52**(D1), D791-D797.

Aghili, F., Jansa, J., Khoshgoftarmanesh, A. H., Afyuni, M., Schulin, R., Frossard, E. & Gamper, H. A. (2014). Wheat plants invest more in mycorrhizae and receive more bene fits from them under adverse than favorable soil conditions. *Applied Soil Ecology* **84**, 93–111.

Aguiar-Pulido, V., Huang, W., Suarez-Ulloa, V., Cickovski, T., Mathee, K. & Narasimhan, G. (2016). Metagenomics, Metatranscriptomics, and Metabolomics Approaches for Microbiome Analysis:Supplementary Issue: Bioinformatics Methods and Applications for Big Metagenomics Data. *Evolutionary Bioinformatics* **12s1**, EBO.S36436.

Albornoz, F. E., Prober, S. M., Ryan, M. H. & Standish, R. J. (2022). Ecological interactions among microbial functional guilds in the plant-soil system and implications for ecosystem function. *Plant and Soil*, 1-13.

Alfonzetti, M., Tetu, S. G., Mills, C. H. & Gallagher, R. V. (2023). Assessing the efficacy of extruded seed pellets and microbial amendments for native revegetation. *Restoration Ecology* **31**(3), e13857.

Alivisatos, A. P., Blaser, M., Brodie, E. L., Chun, M., Dangl, J. L., Donohue, T. J., Dorrestein, P. C., Gilbert, J. A., Green, J. L. & Jansson, J. K. (2015). A unified initiative to harness Earth's microbiomes. *Science* **350**(6260), 507-508.

Allison, S. D. & Martiny, J. B. H. (2008). Resistance, resilience, and redundancy in microbial communities. *Proceedings of the National Academy of Sciences* **105**(supplement_1), 11512-11519.

Amarasinghe, A., Chen, C., Van Zwieten, L. & Rashti, M. R. (2024). The role of edaphic variables and management practices in regulating soil microbial resilience to drought - A meta-analysis. *Science of The Total Environment* **912**, 169544.

Anthony, M., Stinson, K., Trautwig, A., Coates-Connor, E. & Frey, S. (2019). Fungal communities do not recover after removing invasive Alliaria petiolata (garlic mustard). *Biological Invasions* **21**(10), 3085-3099.

ANTHONY, M. A., BENDER, S. F. & VAN DER HEIJDEN, M. G. A. (2023). Enumerating soil biodiversity. *Proceedings of the National Academy of Sciences* **120**(33), e2304663120.

ARADOTTIR, A. L. (2012). Turf transplants for restoration of alpine vegetation: does size matter? *Journal of Applied Ecology* **49**(2), 439-446.

ARIF, S. & MACNEIL, M. A. (2023). Applying the structural causal model framework for observational causal inference in ecology. *Ecological Monographs* **93**(1), e1554.

AUSTRALIA, BUSH HERITAGE (2025). Fitz-Stirling Ecological Scorecard vol. 2025. Bush Heritage Australia. Available at: https://www.bushheritage.org.au/uploads/documents/reserve-scorecards/Fitz-Stirling_scorecard_2017-22.pdf [Accessed 27 March 2025]

AYUSO, S. V., SILVA, A. G., NELSON, C., BARGER, N. N. & GARCIA-PICHEL, F. (2017). Microbial Nursery Production of High-Quality Biological Soil Crust Biomass for Restoration of Degraded Dryland Soils. *Applied and Environmental Microbiology* **83**(3), e02179-16.

BAAS-BECKING, L. G. M. (1934). *Geobiologie; of inleiding tot de milieukunde*. WP Van Stockum & Zoon NV.

BACILIO, M., HERNANDEZ, J. P. & BASHAN, Y. (2006). Restoration of giant cardon cacti in barren desert soil amended with common compost and inoculated with Azospirillum brasilense. *Biology and Fertility of Soils* **43**(1), 112-119.

BANNING, N. C., GLEESON, D. B., GRIGG, A. H., GRANT, C. D., ANDERSEN, G. L., BRODIE, E. L. & MURPHY, D. (2011). Soil microbial community successional patterns during forest ecosystem restoration. *Applied Environmental Microbiology* **77**(17), 6158-6164.

BARBER, N. A., CHANTOS‐DAVIDSON, K. M., AMEL PERALTA, R., SHERWOOD, J. P. & SWINGLEY, W. D. (2017). Soil microbial community composition in tallgrass prairie restorations converge with remnants across a 27‐year chronosequence. *Environmental Microbiology* **19**(8), 3118-3131.

BARBER, N. A., KLIMEK, D. M., BELL, J. K. & SWINGLEY, W. D. (2023). Restoration age and reintroduced bison may shape soil bacterial communities in restored tallgrass prairies. *FEMS Microbiology Ecology* **99**(3).

BASHAN, Y., SALAZAR, B. G., MORENO, M., LOPEZ, B. R. & LINDERMAN, R. G. (2012). Restoration of eroded soil in the Sonoran Desert with native leguminous trees using plant growth-promoting microorganisms and limited amounts of compost and water. *Journal of Environmental Management* **102**, 26-36.

BAUER, J., MACK, K. & BEVER, J. (2015). Plant-soil feedbacks as drivers of succession: evidence from remnant and restored tallgrass prairies. *Ecosphere* 6: art158.

Bell, J. K., Siciliano, S. D. & Lamb, E. G. (2020). A survey of invasive plants on grassland soil microbial communities and ecosystem services. *Scientific Data* **7**(1), 1-8.

Berendsen, R. L., Vismans, G., Yu, K., Song, Y., de Jonge, R., Burgman, W. P., Burmølle, M., Herschend, J., Bakker, P. A. H. M. & Pieterse, C. M. J. (2018). Disease-induced assemblage of a plant-beneficial bacterial consortium. *The ISME Journal* **12**(6), 1496-1507.

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M.-C. C., Charles, T., Chen, X., Cocolin, L., Eversole, K. & Corral, G. H. (2020). Microbiome definition re-visited: old concepts and new challenges. *Microbiome* **8**(1), 1-22.

Berruti, A., Lumini, E., Balestrini, R. & Bianciotto, V. (2016). Arbuscular Mycorrhizal Fungi as Natural Biofertilizers: Let's Benefit from Past Successes. *Frontiers in Microbiology* **6**.

Bever, J. D., Mangan, S. A. & Alexander, H. M. (2015). Maintenance of Plant Species Diversity by Pathogens. *Annual Review of Ecology, Evolution and Systematics* **46**(Volume 46, 2015), 305-325.

Bissett, A., Fitzgerald, A., Meintjes, T., Mele, P. M., Reith, F., Dennis, P. G., Breed, M. F., Brown, B., Brown, M. V. & Brugger, J. (2016). Introducing BASE: the Biomes of Australian Soil Environments soil microbial diversity database. *GigaScience* **5**(1), s13742-016-0126-5.

Blakemore, R. J. (2025). Biodiversity restated: > 99.9% of global species in Soil Biota. *ZooKeys* **1224**.

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., Huttley, G. A. & Gregory Caporaso, J. (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* **6**(1), 90.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., Cope, E. K., Da Silva, R., Diener, C., Dorrestein, P. C., Douglas, G. M., Durall, D. M., Duvallet, C., Edwardson, C. F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J. M., Gibbons, S. M., Gibson, D. L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G. A., Janssen, S., Jarmusch, A. K., Jiang, L., Kaehler, B. D., Kang, K. B., Keefe, C. R., Keim, P., Kelley, S. T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M. G. I., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B. D., McDonald, D., McIver, L. J., Melnik, A. V., Metcalf, J. L., Morgan, S. C., Morton, J. T., Naimey, A. T., Navas-Molina, J. A., Nothias, L. F., Orchanian, S. B., Pearson, T., Peoples, S. L., Petras, D., Preuss, M. L., Pruesse, E., Rasmussen, L. B., Rivers, A., Robeson, M. S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S. J., Spear, J. R., Swafford, A. D., Thompson, L. R., Torres, P. J., Trinh, P.,

Tripathi, A., Turnbaugh, P. J., Ul-Hasan, S., van der Hooft, J. J. J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K. C., Williamson, C. H. D., Willis, A. D., Xu, Z. Z., Zaneveld, J. R., Zhang, Y., Zhu, Q., Knight, R. & Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology* **37**(8), 852-857.

Boyer, S., Wratten, S., Pizey, M. & Weber, P. (2011). Impact of soil stockpiling and mining rehabilitation on earthworm communities. *Pedobiologia* **54**, S99-S102.

Bradby, K., Keesing, A. & Wardell‐Johnson, G. (2016). Gondwana Link: connecting people, landscapes, and livelihoods across southwestern Australia. *Restoration Ecology* **24**(6), 827-835.

Breed, M. F., Harrison, P. A., Blyth, C., Byrne, M., Gaget, V., Gellie, N. J. C., Groom, S. V. C., Hodgson, R., Mills, J. G., Prowse, T. A. A., Steane, D. A. & Mohr, J. J. (2019). The potential of genomics for restoring ecosystems and biodiversity. *Nature Reviews Genetics* **20**(10), 615-628.

Broadhurst, L. M., Jones, T. A., Smith, F. S., North, T. & Guja, L. (2016). Maximizing seed resources for restoration in an uncertain future. *BioScience* **66**(1), 73-79.

Broadhurst, L. M., Prober, S. M., Boggs, G., Bush, D., Breed, M. F., Dickson, F., Harrison, P. A., Jellinek, S., Lynch, A. J. J., Rymer, P. D., Young, R. E. & Commander, L. E. (2023). *Guidelines for embedded experiments in ecological restoration and management in Australia*. CSIRO.

Brown, V. S., Erickson, T. E., Merritt, D. J., Madsen, M. D., Hobbs, R. J. & Ritchie, A. L. (2021). A global review of seed enhancement technology use to inform improved applications in restoration. *Science of The Total Environment* **798**, 149096.

Brudvig, L. A., Barak, R. S., Bauer, J. T., Caughlin, T. T., Laughlin, D. C., Larios, L., Matthews, J. W., Stuble, K. L., Turley, N. E. & Zirbel, C. R. (2017). Interpreting variation to advance predictive restoration science. *Journal of Applied Ecology* **54**(4), 1018-1027.

Brudvig, L. A., Turley, N. E., Bartel, S. L., Bell-Dereske, L., Breland, S., Damschen, E. I., Evans, S. E., Gibbs, J., Hahn, P. G., Isaacs, R., Ledvina, J. A., Orrock, J. L., Sorenson, Q. M. & Stuhler, J. D. (2021). Large ecosystem-scale effects of restoration fail to mitigate impacts of land-use legacies in longleaf pine savannas. *Proceedings of the National Academy of Sciences* **118**(17), e2020935118.

Buchenau, N., van Kleunen, M. & Wilschut, R. A. (2022). Direct and legacy-mediated drought effects on plant performance are species-specific and depend on soil community composition. *Oikos* **2022**(5), e08959.

Buchfink, B., Reuter, K. & Drost, H.-G. (2021). Sensitive protein alignments at tree-of-life scale using DIAMOND. *Nature Methods* **18**(4), 366-368.

BULLOCK, J. M. (1998). Community translocation in Britain: Setting objectives and measuring consequences. *Biological Conservation* **84**(3), 199-214.

BUTT, K. R., GILBERT, J. A., KOSTECKA, J., LOWE, C. N., QUIGG, S. M. & EUTENEUER, P. (2022). Two decades of monitoring earthworms in translocated grasslands at Manchester Airport. *European Journal of Soil Biology* **113**, 103443.

CALLAHAN, B. J., MCMURDIE, P. J., ROSEN, M. J., HAN, A. W., JOHNSON, A. J. A. & HOLMES, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods* **13**(7), 581-583.

CARBAJO, V., DEN BRABER, B., VAN DER PUTTEN, W. H. & DE DEYN, G. B. (2011). Enhancement of late successional plants on ex-arable land by soil inoculations. *PloS one* **6**(7), e21943-e21943.

CAVICCHIOLI, R., RIPPLE, W. J., TIMMIS, K. N., AZAM, F., BAKKEN, L. R., BAYLIS, M., BEHRENFELD, M. J., BOETIUS, A., BOYD, P. W., CLASSEN, A. T., CROWTHER, T. W., DANOVARO, R., FOREMAN, C. M., HUISMAN, J., HUTCHINS, D. A., JANSSON, J. K., KARL, D. M., KOSKELLA, B., MARK WELCH, D. B., MARTINY, J. B. H., MORAN, M. A., ORPHAN, V. J., REAY, D. S., REMAIS, J. V., RICH, V. I., SINGH, B. K., STEIN, L. Y., STEWART, F. J., SULLIVAN, M. B., VAN OPPEN, M. J. H., WEAVER, S. C., WEBB, E. A. & WEBSTER, N. S. (2019). Scientists' warning to humanity: microorganisms and climate change. *Nature Reviews Microbiology* **17**(9), 569-586.

CEBALLOS, G., EHRLICH, P. R., BARNOSKY, A. D., GARCÍA, A., PRINGLE, R. M. & PALMER, T. M. (2015). Accelerated modern human–induced species losses: Entering the sixth mass extinction. *Science Advances* **1**(5), e1400253.

CHAZDON, R. L. (2008). Beyond deforestation: restoring forests and ecosystem services on degraded lands. *Science* **320**(5882), 1458-1460.

CHAZDON, R. L., LINDENMAYER, D., GUARIGUATA, M. R., CROUZEILLES, R., BENAYAS, J. M. R. & CHAVERO, E. L. (2020). Fostering natural forest regeneration on former agricultural land through economic and policy interventions. *Environmental Research Letters* **15**(4), 043002.

CHEN, S., ZHOU, Y., CHEN, Y. & GU, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* **34**(17), i884-i890.

CHEN, W., JIAO, S., LI, Q. & DU, N. (2020). Dispersal limitation relative to environmental filtering governs the vertical small-scale assembly of soil microbiomes during restoration. *Journal of Applied Ecology* **57**(2), 402-412.

CHOUDOIR, M. J. & DEANGELIS, K. M. (2022). A framework for integrating microbial dispersal modes into soil ecosystem ecology. *iScience* **25**(3).

CHRISTIE, A. P., AMANO, T., MARTIN, P. A., SHACKELFORD, G. E., SIMMONS, B. I. & SUTHERLAND, W. J. (2019). Simple study designs in ecology produce inaccurate estimates of biodiversity responses. *Journal of Applied Ecology* **56**(12), 2742-2754.

CHUA, M., ERICKSON, T. E., MERRITT, D. J., CHILTON, A. M., OOI, M. K. J. & MUÑOZ-ROJAS, M. (2019). Bio-priming seeds with cyanobacteria: effects on native plant growth and soil properties. *Restoration Ecology* **n/a**(n/a).

COBAN, O., DE DEYN, G. B. & VAN DER PLOEG, M. (2022). Soil microbiota as game-changers in restoration of degraded lands. *Science* **375**(6584), abe0725.

COLLEN, B. & NICHOLSON, E. (2014). Taking the measure of change. *Science* **346**(6206), 166-167.

CONDRON, L. M., TURNER, B. L. & CADE-MENUN, B. J. (2005). Chemistry and Dynamics of Soil Organic Phosphorus. In *Phosphorus: Agriculture and the Environment*. (pp. 87-121).

CONTOS, P., WOOD, J. L., MURPHY, N. P. & GIBB, H. (2021). Rewilding with invertebrates and microbes to restore ecosystems: Present trends and future directions. *Ecology and Evolution* **11**(12), 7187-7200.

CORDIER, T., LANZÉN, A., APOTHÉLOZ-PERRET-GENTIL, L., STOECK, T. & PAWLOWSKI, J. (2019). Embracing Environmental Genomics and Machine Learning for Routine Biomonitoring. *Trends in Microbiology* **27**(5), 387-397.

COSTANTINI, E. A., BRANQUINHO, C., NUNES, A., SCHWILCH, G., STAVI, I., VALDECANTOS, A. & ZUCCA, C. (2016). Soil indicators to assess the effectiveness of restoration strategies in dryland ecosystems. *Solid Earth* **7**(2), 397-414.

CRAMER, V. A., HOBBS, R. J. & STANDISH, R. J. (2008). What's new about old fields? Land abandonment and ecosystem assembly. *Trends in Ecology & Evolution* **23**(2), 104-112.

CROUZEILLES, R., CURRAN, M., FERREIRA, M. S., LINDENMAYER, D. B., GRELLE, C. E. & BENAYAS, J. M. R. (2016). A global meta-analysis on the ecological drivers of forest restoration success. *Nature Communications* **7**(1), 1-8.

DADZIE, F. A., MOLES, A. T., ERICKSON, T. E., MACHADO DE LIMA, N. & MUÑOZ-ROJAS, M. (2024). Inoculating native microorganisms improved soil function and altered the microbial composition of a degraded soil. *Restoration Ecology* **n/a**(n/a), e14025.

DADZIE, F. A., MOLES, A. T., ERICKSON, T. E., SLAVICH, E. & MUÑOZ‐ROJAS, M. (2022). Native bacteria and cyanobacteria can influence seedling emergence and growth of native plants used in dryland restoration. *Journal of Applied Ecology* **59**(12), 2983-2992.

DAVIES, T., CANDO-DUMANCELA, C., LIDDICOAT, C., DRESKEN, R., DAMEN, R. H., EDWARDS, R. A., RAMESH, S. A. & BREED, M. F. (2024). Ecological phage therapy: Can bacteriophages help rapidly restore the soil microbiome? *Ecology and Evolution* **14**(8), e70185.

DAWS, M. I., STANDISH, R. J., KOCH, J. M., MORALD, T. K., TIBBETT, M. & HOBBS, R. J. (2015). Phosphorus fertilisation and large legume species affect jarrah forest restoration after bauxite mining. *Forest Ecology and Management* **354**, 10-17.

Debray, R., Herbert, R. A., Jaffe, A. L., Crits-Christoph, A., Power, M. E. & Koskella, B. (2021). Priority effects in microbiome assembly. *Nature Reviews Microbiology*, 1-13.

Delgado-Baquerizo, M. & Eldridge, D. J. (2019). Cross-biome drivers of soil bacterial alpha diversity on a worldwide scale. *Ecosystems* **22**(6), 1220-1231.

Delgado-Baquerizo, M., Maestre, F. T., Reich, P. B., Jeffries, T. C., Gaitan, J. J., Encinar, D., Berdugo, M., Campbell, C. D. & Singh, B. K. (2016). Microbial diversity drives multifunctionality in terrestrial ecosystems. *Nature Communications* **7**, 10541.

Delgado-Baquerizo, M., Reich, P. B., Bardgett, R. D., Eldridge, D. J., Lambers, H., Wardle, D. A., Reed, S. C., Plaza, C., Png, G. K. & Neuhauser, S. (2020). The influence of soil age on ecosystem structure and function across biomes. *Nature Communications* **11**(1), 1-14.

Delgado-Baquerizo, M., Reich, P. B., Khachane, A. N., Campbell, C. D., Thomas, N., Freitag, T. E., Abu Al-Soud, W., Sørensen, S., Bardgett, R. D. & Singh, B. K. (2017). It is elemental: soil nutrient stoichiometry drives bacterial diversity. *Environmental Microbiology* **19**(3), 1176-1188.

Diaz-Colunga, J., Lu, N., Sanchez-Gorostiaga, A., Chang, C.-Y., Cai, H. S., Goldford, J. E., Tikhonov, M. & Sánchez, Á. (2022). Top-down and bottom-up cohesiveness in microbial community coalescence. *Proceedings of the National Academy of Sciences* **119**(6), e2111261119.

Dirzo, R. & Raven, P. H. (2003). Global state of biodiversity and loss. *Annual Review of Environment and Resources* **28**.

Domínguez-Castillo, C., Alatorre-Cruz, J. M., Castañeda-Antonio, D., Munive, J. A., Guo, X., López-Olguín, J. F., Fuentes-Ramírez, L. E. & Carreño-López, R. (2021). Potential seed germination-enhancing plant growth-promoting rhizobacteria for restoration of Pinus chiapensis ecosystems. *Journal of Forestry Research* **32**(5), 2143-2153.

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., Huttenhower, C. & Langille, M. G. (2020). PICRUSt2 for prediction of metagenome functions. *Nature Biotechnology* **38**(6), 685-688.

Dutta, S., Na, C. S. & Lee, Y. H. (2021). Features of Bacterial Microbiota in the Wild Habitat of Pulsatilla tongkangensis, the Endangered “Long-Sepal Donggang Pasque-Flower Plant,” Endemic to Karst Topography of Korea. *Frontiers in Microbiology* **12**.

Eisenhauer, N., Antunes, P. M., Bennett, A. E., Birkhofer, K., Bissett, A., Bowker, M. A., Caruso, T., Chen, B., Coleman, D. C. & De Boer, W. (2017a). Priorities for research in soil ecology. *Pedobiologia* **63**, 1-7.

Eisenhauer, N., Bender, S. F., Calderón-Sanou, I., de Vries, F. T., Lembrechts, J. J., Thuiller, W., Wall, D. H., Zeiss, R., Bahram, M., Beugnon, R., Burton, V. J., Crowther, T. W., Delgado-Baquerizo, M., Geisen, S., Kardol, P.,

KRASHEVSKA, V., MARTÍNEZ-MUÑOZ, C. A., PATOINE, G., SEEBER, J., SOUDZILOVSKAIA, N. A., STEINWANDTER, M., SÜNNEMANN, M., SUN, X., VAN DER HEIJDEN, M. G. A., GUERRA, C. A. & POTAPOV, A. (2022). Frontiers in soil ecology—Insights from the World Biodiversity Forum 2022. *Journal of Sustainable Agriculture and Environment* **1**(4), 245-261.

EISENHAUER, N., BOWKER, M. A., GRACE, J. B. & POWELL, J. R. (2015). From patterns to causal understanding: Structural equation modeling (SEM) in soil ecology. *Pedobiologia* **58**(2), 65-72.

EISENHAUER, N., LANOUE, A., STRECKER, T., SCHEU, S., STEINAUER, K., THAKUR, M. P. & MOMMER, L. (2017b). Root biomass and exudates link plant diversity with soil bacterial and fungal biomass. *Scientific Reports* **7**(1), 44641.

ERICKSON, T. E., KILDISHEVA, O. A., BAUGHMAN, O. W., BREED, M. F., RUIZ-TALONIA, L., BROWN, V. S., MADSEN, M. D., MERRITT, D. J. & RITCHIE, A. L. (2021). Florabank guidelines module 12: seed enhancement technologies. In *Florabank Guidelines: best practice guidelines for native seed collection and use*. (Florabank Consortium).

EVINER, V. T. & HAWKES, C. V. (2008). Embracing variability in the application of plant–soil interactions to the restoration of communities and ecosystems. *Restoration Ecology* **16**(4), 713-729.

FAHEY, C. & FLORY, S. L. (2022). Soil microbes alter competition between native and invasive plants. *Journal of Ecology* **110**(2), 404-414.

FARRELL, H. L., LÉGER, A., BREED, M. F. & GORNISH, E. S. (2020). Restoration, Soil Organisms, and Soil Processes: Emerging Approaches. *Restoration Ecology*.

FIELD, K. J. & PRESSEL, S. (2018). Unity in diversity: structural and functional insights into the ancient partnerships between plants and fungi. *New Phytologist* **220**(4), 996-1011.

FIERER, N. (2017). Embracing the unknown: disentangling the complexities of the soil microbiome. *Nature Reviews Microbiology* **15**(10), 579.

FIERER, N. & JACKSON, R. B. (2006). The diversity and biogeography of soil bacterial communities. *Proceedings of the National Academy of Sciences* **103**(3), 626-631.

FIERER, N., WOOD, S. A. & DE MESQUITA, C. P. B. (2021). How microbes can, and cannot, be used to assess soil health. *Soil Biology and Biochemistry* **153**, 108111.

FLINN, K. M. & VELLEND, M. (2005). Recovery of forest plant communities in post-agricultural landscapes. *Frontiers in Ecology and the Environment* **3**(5), 243-250.

FOLEY, J. A., DEFRIES, R., ASNER, G. P., BARFORD, C., BONAN, G., CARPENTER, S. R., CHAPIN, F. S., COE, M. T., DAILY, G. C. & GIBBS, H. K. (2005). Global consequences of land use. *Science* **309**(5734), 570-574.

GANN, G. D., MCDONALD, T., WALDER, B., ARONSON, J., NELSON, C. R., JONSON, J., HALLETT, J. G., EISENBERG, C., GUARIGUATA, M. R. & LIU, J. (2019a). International principles and standards for the practice of ecological restoration. *Restoration Ecology* **27**(S1), S1-S46.

GANN, G. D., MCDONALD, T., WALDER, B., ARONSON, J., NELSON, C. R., JONSON, J., HALLETT, J. G., EISENBERG, C., GUARIGUATA, M. R., LIU, J., HUA, F., ECHEVERRÍA, C., GONZALES, E., SHAW, N., DECLEER, K. & DIXON, K. W. (2019b). International principles and standards for the practice of ecological restoration. Second edition. *Restoration Ecology* **27**(S1), S1-S46.

GANNETT, M., DITOMMASO, A., SPARKS, J. P. & KAO-KNIFFIN, J. (2024). Microbial nitrogen immobilization as a tool to manage weeds in agroecosystems. *Agriculture, Ecosystems & Environment* **366**, 108904.

GELLIE, N. J., BREED, M. F., MORTIMER, P. E., HARRISON, R. D., XU, J. & LOWE, A. J. (2018). Networked and embedded scientific experiments will improve restoration outcomes. *Frontiers in Ecology and the Environment* **16**(5), 288-294.

GELLIE, N. J., MILLS, J. G., BREED, M. F. & LOWE, A. J. (2017). Revegetation rewilds the soil bacterial microbiome of an old field. *Molecular Ecology* **26**(11), 2895-2904.

GERRITS, G. M., WAENINK, R., ARADOTTIR, A. L., BUISSON, E., DUTOIT, T., FERREIRA, M. C., FONTAINE, J. B., JAUNATRE, R., KARDOL, P., LOEB, R., MAGRO RUIZ, S., MALTZ, M., PÄRTEL, M., PECO, B., PIQUERAY, J., PILON, N. A. L., SANTA-REGINA, I., SCHMIDT, K. T., SENGL, P., VAN DIGGELEN, R., VIEIRA, D. L. M., VON BRACKEL, W., WARYSZAK, P., WILLS, T. J., MARRS, R. H. & WUBS, E. R. J. (2023). Synthesis on the effectiveness of soil translocation for plant community restoration. *Journal of Applied Ecology* **60**(4), 714-724.

GIBBS, H. & SALMON, J. M. (2015). Mapping the world's degraded lands. *Applied geography* **57**, 12-21.

GIBSON-ROY, P., DELPRATT, J. & MOORE, G. (2024). Native establishment improved and weed competition reduced by topsoil removal in direct-sown native grasslands. *Ecological Management & Restoration* **25**(1), 68-82.

GIOIA, P. & HOPPER, S. D. (2017). A new phytogeographic map for the Southwest Australian Floristic Region after an exceptional decade of collection and discovery. *Botanical Journal of the Linnean Society* **184**(1), 1-15.

GOMES, S. I. F., GUNDERSEN, P., BEZEMER, T. M., BARSOTTI, D., D'IMPERIO, L., GEORGOPOULOS, K., JUSTESEN, M. J., RHEAULT, K., ROSAS, Y. M., SCHMIDT, I. K., TEDERSOO, L., VESTERDAL, L., YU, M., ANSLAN, S., ASLANI, F., BYRIEL, D. B., CHRISTIANSEN, J., HANSEN, S. H., KASAL, N., KOSAWANG, C., LARSEN, H., LARSEN, K. S., LEES, J., VAN DIJKE, A. C. P. & KEPFER-ROJAS, S. (2025). Soil Microbiome Inoculation for Resilient and Multifunctional New Forests in Post-Agricultural Landscapes. *Global Change Biology* **31**(1), e70031.

GOPAL, M. & GUPTA, A. (2016). Microbiome selection could spur next-generation plant breeding strategies. *Frontiers in Microbiology* **7**, 1971.

GORNISH, E., ARNOLD, H. & FEHMI, J. (2019). Review of seed pelletizing strategies for arid land restoration. *Restoration Ecology* **27**(6), 1206-1211.

GORNISH, E. S., FRANKLIN, K., ROWE, J. & BARBERÁN, A. (2020). Buffelgrass invasion and glyphosate effects on desert soil microbiome communities. *Biological Invasions*, 1-11.

GRACE, J. B. & IRVINE, K. M. (2020). Scientist's guide to developing explanatory statistical models using causal analysis principles. *Ecology* **101**(4), e02962.

GUEVARA-TORRES, D. R., ZAKRZEWSKI, M. & FACELLI, J. M. (2024). Soil scalping and soil microbial filtrates effects on the restoration of temperate grasslands. *Restoration Ecology* **n/a**(n/a), e14351.

HAN, X., LI, Y., LI, Y., DU, X., LI, B., LI, Q. & BEZEMER, T. M. (2022). Soil inoculum identity and rate jointly steer microbiomes and plant communities in the field. *ISME Communications* **2**(1).

HARRIS, J. (2009). Soil microbial communities and restoration ecology: facilitators or followers? *Science* **325**(5940), 573-574.

HENEGHAN, L., MILLER, S. P., BAER, S., CALLAHAM JR, M. A., MONTGOMERY, J., PAVAO‐ZUCKERMAN, M., RHOADES, C. C. & RICHARDSON, S. (2008). Integrating soil ecological knowledge into restoration management. *Restoration Ecology* **16**(4), 608-617.

HERNANDEZ, J. A. C., RIBEIRO, H. M., BAYNE, E., MACKENZIE, M. D. & LANOIL, B. D. (2024). Impact of stockpile depth and storage time on soil microbial communities. *Applied Soil Ecology* **196**, 105275.

HIGGS, E., HARRIS, J., MURPHY, S., BOWERS, K., HOBBS, R., JENKINS, W., KIDWELL, J., LOPOUKHINE, N., SOLLEREDER, B. & SUDING, K. (2018). On principles and standards in ecological restoration. *Restoration Ecology* **26**(3), 399-403.

HIJMANS, R. J., WILLIAMS, E., VENNES, C. & HIJMANS, M. R. J. (2017). Package 'geosphere'. *Spherical Trigonometry* **1**, 7.

HOBBS, R. (2009). Looking for the Silver Lining: Making the Most of Failure. *Restoration Ecology* **17**(1), 1-3.

HOBBS, R. J. & NORTON, D. A. (1996). Towards a conceptual framework for restoration ecology. *Restoration Ecology* **4**(2), 93-110.

HOBBS, R. J. & NORTON, D. A. (2004). Ecological filters, thresholds, and gradients in resistance to ecosystem reassembly. *Assembly rules and restoration ecology: bridging the gap between theory and practice*, 72-95.

HOPPER, S. D. (2009). OCBIL theory: towards an integrated understanding of the evolution, ecology and conservation of biodiversity on old, climatically buffered, infertile landscapes. *Plant and Soil* **322**(1), 49-86.

HOPPER, S. D. & GIOIA, P. (2004). The Southwest Australian Floristic Region: Evolution and Conservation of a Global Hot Spot of Biodiversity. *Annual Review of Ecology, Evolution, and Systematics* **35**(1), 623-650.

HOPPER, S. D., SILVEIRA, F. A. & FIEDLER, P. L. (2016). Biodiversity hotspots and Ocbil theory. *Plant and Soil* **403**(1), 167-216.

HOSSAIN, M. M. (2022). Orchid mycorrhiza: Isolation, culture, characterization and application. *South African Journal of Botany* **151**, 365-384.

HULVEY, K. B., LEGER, E. A., PORENSKY, L. M., ROCHE, L. M., VEBLEN, K. E., FUND, A., SHAW, J. & GORNISH, E. S. (2017). Restoration islands: a tool for efficiently restoring dryland ecosystems? *Restoration Ecology* **25**, S124-S134.

IFTIKHAR, A., FAROOQ, R., AKHTAR, M., KHALID, H., HUSSAIN, N., ALI, Q., MALOOK, S. U. & ALI, D. (2024). Ecological and sustainable implications of phosphorous-solubilizing microorganisms in soil. *Discover Applied Sciences* **6**(2), 33.

JANGID, K., WILLIAMS, M. A., FRANZLUEBBERS, A. J., SCHMIDT, T. M., COLEMAN, D. C. & WHITMAN, W. B. (2011). Land-use history has a stronger impact on soil microbial community composition than aboveground vegetation and soil properties. *Soil Biology and Biochemistry* **43**(10), 2184-2193.

JIANG, M., DELGADO-BAQUERIZO, M., YUAN, M. M., DING, J., YERGEAU, E., ZHOU, J., CROWTHER, T. W. & LIANG, Y. (2023). Home-based microbial solution to boost crop growth in low-fertility soil. *New Phytologist* **239**(2), 752-765.

JIN, C. W., YE, Y. Q. & ZHENG, S. J. (2013). An underground tale: contribution of microbial activity to plant iron acquisition via ecological processes. *Annals of Botany* **113**(1), 7-18.

JONSON, J. (2010). Ecological restoration of cleared agricultural land in Gondwana Link: lifting the bar at 'Peniup'. *Ecological Management & Restoration* **11**(1), 16-26.

JOST, L. (2006). Entropy and diversity. *Oikos* **113**(2), 363-375.

KAISERMANN, A., DE VRIES, F. T., GRIFFITHS, R. I. & BARDGETT, R. D. (2017). Legacy effects of drought on plant–soil feedbacks and plant–plant interactions. *New Phytologist* **215**(4), 1413-1424.

KAMINSKY, L. M., TREXLER, R. V., MALIK, R. J., HOCKETT, K. L. & BELL, T. H. (2019). The Inherent Conflicts in Developing Soil Microbial Inoculants. *Trends in Biotechnology* **37**(2), 140-151.

KARDOL, P., BEZEMER, T. M. & VAN DER PUTTEN, W. H. (2009). Soil Organism and Plant Introductions in Restoration of Species-Rich Grassland Communities. *Restoration ecology* **17**(2), 258-269.

KARDOL, P., MARTIJN BEZEMER, T. & VAN DER PUTTEN, W. H. (2006). Temporal variation in plant–soil feedback controls succession. *Ecology letters* **9**(9), 1080-1088.

KARDOL, P. & WARDLE, D. A. (2010). How understanding aboveground–belowground linkages can assist restoration ecology. *Trends in ecology & evolution* **25**(11), 670-679.

KING, W. L. & BELL, T. H. (2022). Can dispersal be leveraged to improve microbial inoculant success? *Trends in Biotechnology* **40**(1), 12-21.

KLEBER, M., EUSTERHUES, K., KEILUWEIT, M., MIKUTTA, C., MIKUTTA, R. & NICO, P. S. (2015). Chapter One - Mineral–Organic Associations: Formation, Properties, and Relevance in Soil Environments. In *Advances in Agronomy*. (Volume 130, ed D. L. Sparks), pp. 1-140. Academic Press.

KLOPF, R. P., BAER, S. G., BACH, E. M. & SIX, J. (2017). Restoration and management for plant diversity enhances the rate of belowground ecosystem recovery. *Ecological applications* **27**(2), 355-362.

KOS, M., TUIJL, M. A. B., DE ROO, J., MULDER, P. P. J. & BEZEMER, T. M. (2015). Species-specific plant–soil feedback effects on above-ground plant–insect interactions. *Journal of Ecology* **103**(4), 904-914.

KOZIOL, L., CREWS, T. E. & BEVER, J. D. (2020). Native plant abundance, diversity, and richness increases in prairie restoration with field inoculation density of native mycorrhizal amendments. *Restoration ecology*.

KOZIOL, L., SCHULTZ, P. A., HOUSE, G. L., BAUER, J. T., MIDDLETON, E. L. & BEVER, J. D. (2018). The Plant Microbiome and Native Plant Restoration: The Example of Native Mycorrhizal Fungi. *BioScience* **68**(12), 996-1006.

KUHN, M. (2015). Caret: classification and regression training. *Astrophysics Source Code Library*, ascl: 1505.003.

LADOUCEUR, E., JIMÉNEZ‐ALFARO, B., MARIN, M., DE VITIS, M., ABBANDONATO, H., IANNETTA, P. P., BONOMI, C. & PRITCHARD, H. W. (2018). Native seed supply and the restoration species pool. *Conservation Letters* **11**(2), e12381.

LAMBERS, H., AHMEDI, I., BERKOWITZ, O., DUNNE, C., FINNEGAN, P. M., HARDY, G. E. S. J., JOST, R., LALIBERTÉ, E., PEARSE, S. J. & TESTE, F. P. (2013). Phosphorus nutrition of phosphorus-sensitive Australian native plants: threats to plant communities in a global biodiversity hotspot. *Conservation Physiology* **1**(1).

LAMBERS, H., BRUNDRETT, M. C., RAVEN, J. A. & HOPPER, S. D. (2011). Plant mineral nutrition in ancient landscapes: high plant species diversity on infertile soils is linked to functional diversity for nutritional strategies. *Plant and Soil* **348**, 7-27.

LARSON, J. L., VENETTE, R. C. & LARSON, D. L. (2022). Restoration for Resilience: The Role of Plant-Microbial Interactions and Seed Provenance in Ecological Restoration. *Natural Areas Journal* **42**(2), 152-159.

LECLÈRE, D., OBERSTEINER, M., BARRETT, M., BUTCHART, S. H., CHAUDHARY, A., DE PALMA, A., DECLERCK, F. A., DI MARCO, M., DOELMAN, J. C. & DÜRAUER, M. (2020). Bending the curve of terrestrial biodiversity needs an integrated strategy. *Nature* **585**(7826), 551-556.

LEFF, J. W., JONES, S. E., PROBER, S. M., BARBERÁN, A., BORER, E. T., FIRN, J. L., HARPOLE, W. S., HOBBIE, S. E., HOFMOCKEL, K. S., KNOPS, J. M. H., MCCULLEY, R. L., LA PIERRE, K., RISCH, A. C., SEABLOOM, E. W., SCHÜTZ, M., STEENBOCK, C., STEVENS, C. J. & FIERER, N. (2015). Consistent responses of soil microbial communities to elevated nutrient inputs in grasslands across the globe. *Proceedings of the National Academy of Sciences* **112**(35), 10967-10972.

LEM, A. J., LIDDICOAT, C., BISSETT, A., CANDO‐DUMANCELA, C., GARDNER, M. G., PEDDLE, S. D., WATSON, C. D. & BREED, M. F. (2022). Does revegetation cause soil microbiota recovery? Evidence from revisiting a revegetation chronosequence six years after initial sampling. *Restoration ecology*, e13635.

LI, R., TUN, H. M., JAHAN, M., ZHANG, Z., KUMAR, A., DILANTHA FERNANDO, W. G., FARENHORST, A. & KHAFIPOUR, E. (2017). Comparison of DNA-, PMA-, and RNA-based 16S rRNA Illumina sequencing for detection of live bacteria in water. *Scientific reports* **7**(1), 5752.

LIDDICOAT, C., EDWARDS, R. A., ROACH, M., ROBINSON, J. M., WALLACE, K. J., BARNES, A. D., BRAME, J., HEINTZ-BUSCHART, A., CAVAGNARO, T. R., DINSDALE, E. A., DOANE, M. P., EISENHAUER, N., MITCHELL, G., RAI, B., RAMESH, S. & BREED, M. F. (2024). Bioenergetic mapping of 'healthy microbiomes' via compound processing potential imprinted in gut and soil metagenomes. *bioRxiv*, 2023.11.05.565728.

LIDDICOAT, C., KRAUSS, S. L., BISSETT, A., BORRETT, R. J., DUCKI, L. C., PEDDLE, S. D., BULLOCK, P., DOBROWOLSKI, M. P., GRIGG, A., TIBBETT, M. & BREED, M. F. (2022). Next generation restoration metrics: Using soil eDNA bacterial community data to measure trajectories towards rehabilitation targets. *Journal of Environmental Management* **310**, 114748.

LIN, H. & PEDDADA, S. D. (2024). Multigroup analysis of compositions of microbiomes with covariate adjustments and repeated measures. *Nature methods* **21**(1), 83-91.

LIU, L., GUNDERSEN, P., ZHANG, T. & MO, J. (2012). Effects of phosphorus addition on soil microbial biomass and community composition in three forest types in tropical China. *Soil Biology and Biochemistry* **44**(1), 31-38.

LIU, X. & SALLES, J. F. (2024). Drivers and consequences of microbial community coalescence. *The ISME journal* **18**(1).

LOUCA, S., POLZ, M. F., MAZEL, F., ALBRIGHT, M. B. N., HUBER, J. A., O'CONNOR, M. I., ACKERMANN, M., HAHN, A. S., SRIVASTAVA, D. S., CROWE, S. A., DOEBELI, M. & PARFREY, L. W. (2018). Function and functional redundancy in microbial systems. *Nature ecology & evolution* **2**(6), 936-943.

Lu, J., Breitwieser, F. P., Thielen, P. & Salzberg, S. L. (2017). Bracken: estimating species abundance in metagenomics data. *PeerJ Computer Science* **3**, e104.

Lu, J., Rincon, N., Wood, D. E., Breitwieser, F. P., Pockrandt, C., Langmead, B., Salzberg, S. L. & Steinegger, M. (2022). Metagenome analysis using the Kraken software suite. *Nature Protocols* **17**(12), 2815-2839.

Madsen, M. D., Davies, K. W., Boyd, C. S., Kerby, J. D. & Svejcar, T. J. (2016). Emerging seed enhancement technologies for overcoming barriers to restoration. *Restoration ecology* **24**, S77-S84.

Madsen, M. D., Svejcar, L., Radke, J. & Hulet, A. (2018). Inducing rapid seed germination of native cool season grasses with solid matrix priming and seed extrusion technology. *PloS one* **13**(10), e0204380.

Makiola, A., Compson, Z. G., Baird, D. J., Barnes, M. A., Boerlijst, S. P., Bouchez, A., Brennan, G., Bush, A., Canard, E., Cordier, T., Creer, S., Curry, R. A., David, P., Dumbrell, A. J., Gravel, D., Hajibabaei, M., Hayden, B., van der Hoorn, B., Jarne, P., Jones, J. I., Karimi, B., Keck, F., Kelly, M., Knot, I. E., Krol, L., Massol, F., Monk, W. A., Murphy, J., Pawlowski, J., Poisot, T., Porter, T. M., Randall, K. C., Ransome, E., Ravigné, V., Raybould, A., Robin, S., Schrama, M., Schatz, B., Tamaddoni-Nezhad, A., Trimbos, K. B., Vacher, C., Vasselon, V., Wood, S., Woodward, G. & Bohan, D. A. (2020). Key Questions for Next-Generation Biomonitoring. *Frontiers in Environmental Science* **7**(197).

Mansfield, T., Hardy, G., Fleming, P. & Standish, R. (2024). Recruitment failure of keystone trees in Phytophthora infested forest. *Austral Ecology* **49**(2), e13500.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *2011* **17**(1), 3.

Martiny, J. B. H., Bohannan, B. J., Brown, J. H., Colwell, R. K., Fuhrman, J. A., Green, J. L., Horner-Devine, M. C., Kane, M., Krumins, J. A. & Kuske, C. R. (2006). Microbial biogeography: putting microorganisms on the map. *Nature Reviews Microbiology* **4**(2), 102-112.

Mason, C. N., Shahar, S., Beals, K. K., Kelley, S. T., Lipson, D. A., Swingley, W. D. & Barber, N. A. (2023). Taxonomic and functional restoration of tallgrass prairie soil microbial communities in comparison to remnant and agricultural soils. *FEMS Microbiology Ecology* **99**(11).

McBratney, A. B., Mendonça Santos, M. L. & Minasny, B. (2003). On digital soil mapping. *Geoderma* **117**(1), 3-52.

McBratney, A. B., Santos, M. M. & Minasny, B. (2003). On digital soil mapping. *Geoderma* **117**(1-2), 3-52.

McKinley, V. L. (2019). Effects of land use and restoration on soil microbial communities. In *Understanding Terrestrial Microbial Communities*. (pp. 173-242. Springer.

McMurdie, P. J. & Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one* **8**(4), e61217.

Meteorology, Australian Bureau of (2025). Average annual, seasonal and monthly
rainfall. Australian Government. Available at http://www.bom.gov.au/jsp/ncc/cdio/weatherData/av?p_nccObsCode=139&p_display [Accessed 24 March 2025].

Mohr, J. J., Harrison, P. A., Stanhope, J. & Breed, M. F. (2022). Is the genomics 'cart'before the restoration ecology 'horse'? Insights from qualitative interviews and trends from the literature. *Philosophical Transactions of the Royal Society B* **377**(1857), 20210381.

Monks, L., Barrett, S., Beecham, B., Byrne, M., Chant, A., Coates, D., Cochrane, J. A., Crawford, A., Dillon, R. & Yates, C. (2019). Recovery of threatened plant species and their habitats in the biodiversity hotspot of the Southwest Australian Floristic Region. *Plant Diversity* **41**(2), 59-74.

Moradi, J., Vicentini, F., Šimáčková, H., Pižl, V., Tajovský, K., Stary, J. & Frouz, J. (2018). An investigation into the long-term effect of soil transplant in bare spoil heaps on survival and migration of soil meso and macrofauna. *Ecological Engineering* **110**, 158-164.

Moreno-Mateos, D., Alberdi, A., Morriën, E., van der Putten, W. H., Rodríguez-Uña, A. & Montoya, D. (2020). The long-term restoration of ecosystem complexity. *Nature Ecology & Evolution*, 1-10.

Morris, A., Meyer, K. & Bohannan, B. (2020). Linking microbial communities to ecosystem functions: what we can learn from genotype–phenotype mapping in organisms. *Philosophical Transactions of the Royal Society B* **375**(1798), 20190244.

Mueller, U. G. & Sachs, J. L. (2015). Engineering Microbiomes to Improve Plant and Animal Health. *Trends in Microbiology* **23**(10), 606-617.

Muñoz-Rojas, M. (2018). Soil quality indicators: critical tools in ecosystem restoration. *Current Opinion in Environmental Science & Health* **5**, 47-52.

Muñoz-Rojas, M., Chilton, A., Liyanage, G. S., Erickson, T. E., Merritt, D. J., Neilan, B. A. & Ooi, M. K. J. (2018). Effects of indigenous soil cyanobacteria on seed germination and seedling growth of arid species used in restoration. *Plant and Soil* **429**(1), 91-100.

Munro, T. P., Erickson, T. E., Nimmo, D. G., Dadzie, F. A., Muñoz-Rojas, M. & Price, J. N. (2024). Exploring the potential of topsoil pellets to improve native seedling establishment on degraded agricultural land. *Plant and Soil*.

Murphy, C. A., Foster, B. L., Ramspott, M. E. & Price, K. P. (2004). Grassland management effects on soil bulk density. *Transactions of the Kansas Academy of Science* **107**(1), 45-54, 10.

MYERS, N., MITTERMEIER, R. A., MITTERMEIER, C. G., DA FONSECA, G. A. & KENT, J. (2000). Biodiversity hotspots for conservation priorities. *Nature* **403**(6772), 853-858.

NAEEM, S., THOMPSON, L. J., LAWLER, S. P., LAWTON, J. H. & WOODFIN, R. M. (1994). DECLINING BIODIVERSITY CAN ALTER THE PERFORMANCE OF ECOSYSTEMS. *Nature* **368**(6473), 734-737.

NANNIPIERI, P., ASCHER, J., CECCHERINI, M. T., LANDI, L., PIETRAMELLARA, G. & RENELLA, G. (2017). Microbial diversity and soil functions. *European Journal of Soil Science* **68**(1), 12-26.

NAVARRETE, A. A., TSAI, S. M., MENDES, L. W., FAUST, K., DE HOLLANDER, M., CASSMAN, N. A., RAES, J., VAN VEEN, J. A. & KURAMAE, E. E. (2015). Soil microbiome responses to the short-term effects of Amazonian deforestation. *Molecular Ecology* **24**(10), 2433-2448.

NGUGI, M. R., DENNIS, P. G., NELDNER, V. J., DOLEY, D., FECHNER, N. & MCELNEA, A. (2018). Open‐cut mining impacts on soil abiotic and bacterial community properties as shown by restoration chronosequence. *Restoration Ecology* **26**(5), 839-850.

NKONGOLO, K. & NARENDRULA-KOTHA, R. (2020a). Advances in monitoring soil microbial community dynamic and function. *Journal of Applied Genetics* **61**, 249-263.

NKONGOLO, K. & NARENDRULA-KOTHA, R. (2020b). Advances in monitoring soil microbial community dynamic and function. *Journal of Applied Genetics*, 1-15.

O'CALLAGHAN, M. (2016). Microbial inoculation of seed for improved crop performance: issues and opportunities. *Applied Microbiology and Biotechnology* **100**(13), 5729-5746.

OKSANEN, J., BLANCHET, F. G., KINDT, R., LEGENDRE, P., MINCHIN, P. R., O'HARA, R., SIMPSON, G. L., SOLYMOS, P., STEVENS, M. H. H. & WAGNER, H. (2013). Package 'vegan'. *Community ecology package, version* **2**(9), 1-295.

OLIVERIO, A. M., BISSETT, A., MCGUIRE, K., SALTONSTALL, K., TURNER, B. L. & FIERER, N. (2020). The Role of Phosphorus Limitation in Shaping Soil Bacterial Communities and Their Metabolic Capabilities. *mBio* **11**(5), 10.1128/mbio.01718-20.

OLLE, M. & WILLIAMS, I. H. (2013). Effective microorganisms and their influence on vegetable production – a review. *The Journal of Horticultural Science and Biotechnology* **88**(4), 380-386.

OR, D., KELLER, T. & SCHLESINGER, W. H. (2021). Natural and managed soil structure: On the fragile scaffolding for soil functioning. *Soil and Tillage Research* **208**, 104912.

OSBORNE, T., BROCK, S., CHAZDON, R., CHOMBA, S., GAREN, E., GUTIERREZ, V., LAVE, R., LEFEVRE, M. & SUNDBERG, J. (2021). The political ecology playbook for

ecosystem restoration: Principles for effective, equitable, and transformative landscapes. *Global Environmental Change* **70**, 102320.

OSBURN, E. D., AYLWARD, F. O. & BARRETT, J. E. (2021). Historical land use has long-term effects on microbial community assembly processes in forest soils. *ISME Communications* **1**(1).

OVERBEEK, R., DISZ, T. & STEVENS, R. (2004). The SEED: a peer-to-peer environment for genome annotation. *Commun. ACM* **47**(11), 46–51.

PANG, F., LI, Q., SOLANKI, M. K., WANG, Z., XING, Y.-X. & DONG, D.-F. (2024). Soil phosphorus transformation and plant uptake driven by phosphate-solubilizing microorganisms. *Frontiers in Microbiology* **15**.

PARKHURST, T., PROBER, S. M., HOBBS, R. J. & STANDISH, R. J. (2022a). Global meta‐analysis reveals incomplete recovery of soil conditions and invertebrate assemblages after ecological restoration in agricultural landscapes. *Journal of Applied Ecology* **59**(2), 358-372.

PARKHURST, T., STANDISH, R. J. & PROBER, S. M. (2022b). P is for persistence: Soil phosphorus remains elevated for more than a decade after old field restoration. *Ecological Applications* **32**(3), e2547.

PARSONS, L. S., SAYRE, J., ENDER, C., MAZZA RODRIGUES, J. L. & BARBERÁN, A. (2020). Soil Microbial Communities in Restored and Unrestored Coastal Dune Ecosystems in California. *Restoration Ecology*.

PEDDLE, S. D., BISSETT, A., BORRETT, R. J., BULLOCK, P., GARDNER, M. G., LIDDICOAT, C., TIBBETT, M., BREED, M. F. & KRAUSS, S. L. (2022). Soil DNA chronosequence analysis shows bacterial community re‐assembly following post‐mining forest rehabilitation. *Restoration Ecology*, e13706.

PEDDLE, S. D., CANDO-DUMANCELA, C., KRAUSS, S. L., LIDDICOAT, C., SANDERS, A. & BREED, M. F. (2024a). Agricultural land-use legacies affect soil bacterial communities following restoration in a global biodiversity hotspot. *Biological Conservation* **290**, 110437.

PEDDLE, S. D., HODGSON, R. J., BORRETT, R. J., BRACHMANN, S., DAVIES, T. C., ERICKSON, T. E., LIDDICOAT, C., MUÑOZ-ROJAS, M., ROBINSON, J. M., WATSON, C. D., KRAUSS, S. L. & BREED, M. F. (2024b). Practical applications of soil microbiota to improve ecosystem restoration: current knowledge and future directions. *Biological Reviews* **100**(1) (1-18).

PERRING, M. P., ERICKSON, T. E. & BRANCALION, P. H. (2018). Rocketing restoration: enabling the upscaling of ecological restoration in the Anthropocene. *Restoration ecology* **26**(6), 1017-1023.

PERRING, M. P., STANDISH, R. J., PRICE, J. N., CRAIG, M. D., ERICKSON, T. E., RUTHROF, K. X., WHITELEY, A. S., VALENTINE, L. E. & HOBBS, R. J. (2015). Advances in restoration ecology: rising to the challenges of the coming decades. *Ecosphere* **6**(8), 1-25.

PETIPAS, R. H., GEBER, M. A. & LAU, J. A. (2021). Microbe-mediated adaptation in plants. *Ecology Letters* **00**, 1–16.

PHILIPPOT, L., CHENU, C., KAPPLER, A., RILLIG, M. C. & FIERER, N. (2023). The interplay between microbial communities and soil properties. *Nature Reviews Microbiology*.

PINEDA, A., KAPLAN, I. & BEZEMER, T. M. (2017). Steering Soil Microbiomes to Suppress Aboveground Insect Pests. *Trends in Plant Science* **22**(9), 770-778.

PINO, V., MCBRATNEY, A., FAJARDO, M., WILSON, N. & DEAKER, R. (2019). Understanding soil biodiversity using two orthogonal 1000km transects across New South Wales, Australia. *Geoderma* **354**, 113860.

POORE, J. A. C. (2016). Call for conservation: Abandoned pasture. *Science* **351**(6269), 132-132.

PROBER, S. M., LEFF, J. W., BATES, S. T., BORER, E. T., FIRN, J., HARPOLE, W. S., LIND, E. M., SEABLOOM, E. W., ADLER, P. B. & BAKKER, J. D. (2015). Plant diversity predicts beta but not alpha diversity of soil microbes across grasslands worldwide. *Ecology Letters* **18**(1), 85-95.

PROBER, S. M., LIEDLOFF, A. C., ENGLAND, J. R., MOKANY, K., OGILVY, S. & RICHARDS, A. E. (2025). Accounting for the biodiversity benefits of woody plantings in agricultural landscapes: A global meta-analysis. *Agriculture, Ecosystems & Environment* **381**, 109453.

PROSSER, J. I. (2020). Putting science back into microbial ecology: a question of approach. *Philosophical Transactions of the Royal Society B* **375**(1798), 20190240.

QU, X., LI, X., BARDGETT, R. D., KUZYAKOV, Y., REVILLINI, D., SONNE, C., XIA, C., RUAN, H., LIU, Y., CAO, F., REICH, P. B. & DELGADO-BAQUERIZO, M. (2024). Deforestation impacts soil biodiversity and ecosystem services worldwide. *Proceedings of the National Academy of Sciences* **121**(13), e2318475121.

RADHAPRIYA, P., RAMACHANDRAN, A. & PALANI, P. (2018). Indigenous plant growth-promoting bacteria enhance plant growth, biomass, and nutrient uptake in degraded forest plants. *3 Biotech* **8**(3), 154.

RAMAKRISHNA, W., YADAV, R. & LI, K. F. (2019). Plant growth promoting bacteria in agriculture: Two sides of a coin. *Applied Soil Ecology* **138**, 10-18.

RANHEIM SVEEN, T., HANNULA, S. E. & BAHRAM, M. (2024). Microbial regulation of feedbacks to ecosystem change. *Trends in Microbiology* **32**(1), 68-78.

RAUPP, P., CARRILLO, Y. & NIELSEN, U. N. (2024). Soil Health to Enhance Ecological Restoration and Conservation. *Journal of Sustainable Agriculture and Environment* **3**(4), e70022.

Rawat, V. S., Kaur, J., Bhagwat, S., Pandit, M. A. & Rawat, C. D. (2022). Deploying Microbes as Drivers and Indicators in Ecological Restoration. *Restoration Ecology*, e13688.

Rillig, M. C., Lehmann, A., Rongstock, R., Li, H. & Harris, J. (2024). Moving restoration ecology forward with combinatorial approaches. *Global Change Biology* **30**(6), e17361.

Rillig, M. C., Muller, L. A. H. & Lehmann, A. (2017). Soil aggregates as massively concurrent evolutionary incubators. *The ISME Journal* **11**(9), 1943-1948.

Rillig, M. C. & Mummey, D. L. (2006). Mycorrhizas and soil structure. *New Phytologist* **171**(1), 41-53.

Robinson, J. M., Annells, A., Cando-Dumancela, C. & Breed, M. F. (2024a). Sonic restoration: acoustic stimulation enhances plant growth-promoting fungi activity. *Biology Letters* **20**(10), 20240295.

Robinson, J. M., Hodgson, R., Krauss, S. L., Liddicoat, C., Malik, A. A., Martin, B. C., Mohr, J. J., Moreno-Mateos, D., Muñoz-Rojas, M., Peddle, S. D. & Breed, M. F. (2023). Opportunities and challenges for microbiomics in ecosystem restoration. *Trends in Ecology & Evolution*.

Robinson, J. M., Liddicoat, C., Muñoz-Rojas, M. & Breed, M. F. (2024b). Primer: Restoring soil biodiversity. *Current Biology* **34**(R1-R6).

Rodriguez, R. & Durán, P. (2020). Natural Holobiome Engineering by Using Native Extreme Microbiome to Counteract the Climate Change Effects. *Frontiers in Bioengineering and Biotechnology* **8**, 568-568.

Roger-Estrade, J., Anger, C., Bertrand, M. & Richard, G. (2010). Tillage and soil ecology: Partners for sustainable agriculture. *Soil and Tillage Research* **111**(1), 33-40.

Rokich, D. P., Dixon, K. W., Sivasithamparam, K. & Meney, K. A. (2000). Topsoil handling and storage effects on woodland restoration in Western Australia. *Restoration Ecology* **8**(2), 196-208.

Román, J. R., Chilton, A., Cantón, Y. & Muñoz-Rojas, M. (2020). Assessing the viability of cyanobacteria pellets for application in arid land restoration. *Journal of Environmental Management* **270**, 110795.

Román, J. R., Roncero-Ramos, B., Chamizo, S., Rodríguez-Caballero, E. & Cantón, Y. (2018). Restoring soil functions by means of cyanobacteria inoculation: Importance of soil conditions and species selection. *Land Degradation & Development* **29**(9), 3184-3193.

Ruiz‐Jaen, M. C. & Mitchell Aide, T. (2005). Restoration success: how is it being measured? *Restoration Ecology* **13**(3), 569-577.

SAMUELS, T., BRYCE, C., LANDENMARK, H., MARIE-LOUDON, C., NICHOLSON, N., STEVENS, A. H. & COCKELL, C. (2020). Microbial Weathering of Minerals and Rocks in Natural Environments. In *Biogeochemical Cycles*. (59-79).

SCHELFHOUT, S., DE SCHRIJVER, A., DE BOLLE, S., DE GELDER, L., DEMEY, A., DU PRÉ, T., DE NEVE, S., HAESAERT, G., VERHEYEN, K. & MERTENS, J. (2015). Phosphorus mining for ecological restoration on former agricultural land. *Restoration ecology* **23**(6), 842-851.

SCHMID, C. A., REICHEL, R., SCHRÖDER, P., BRÜGGEMANN, N. & SCHLOTER, M. (2020). 52 years of ecological restoration following a major disturbance by opencast lignite mining does not reassemble microbiome structures of the original arable soils. *Science of The Total Environment*, 140955.

SCHNOOR, T. K., LEKBERG, Y., ROSENDAHL, S. & OLSSON, P. A. (2011). Mechanical soil disturbance as a determinant of arbuscular mycorrhizal fungal communities in semi-natural grassland. *Mycorrhiza* **21**(3), 211-220.

SCHOEBITZ, M., LÓPEZ, M. D. & ROLDÁN, A. (2013). Bioencapsulation of microbial inoculants for better soil–plant fertilization. A review. *Agronomy for Sustainable Development* **33**(4), 751-765.

SEYMOUR, M. (2019). Rapid progression and future of environmental DNA research. *Communications Biology* **2**(1), 1-3.

SILLIMAN, B. R., HENSEL, M. J. S., GIBERT, J. P., DALEO, P., SMITH, C. S., WIECZYNSKI, D. J., ANGELINI, C., PAXTON, A. B., ADLER, A. M., ZHANG, Y. S., ALTIERI, A. H., PALMER, T. M., JONES, H. P., GITTMAN, R. K., GRIFFIN, J. N., O'CONNOR, M. I., VAN DE KOPPEL, J., POULSEN, J. R., RIETKERK, M., HE, Q., BERTNESS, M. D., VAN DER HEIDE, T. & VALDEZ, S. R. (2024). Harnessing ecological theory to enhance ecosystem restoration. *Current Biology* **34**(9), R418-R434.

SILVA, G. G. Z., GREEN, K. T., DUTILH, B. E. & EDWARDS, R. A. (2015). SUPER-FOCUS: a tool for agile functional analysis of shotgun metagenomic data. *Bioinformatics* **32**(3), 354-361.

SINGH, J. P., BOTTOS, E. M., VAN HAMME, J. D. & FRASER, L. H. (2024). Microbial composition and function in reclaimed mine sites along a reclamation chronosequence become increasingly similar to undisturbed reference sites. *Science of The Total Environment* **920**, 170996.

SIX, J., BOSSUYT, H., DEGRYZE, S. & DENEF, K. (2004). A history of research on the link between (micro)aggregates, soil biota, and soil organic matter dynamics. *Soil and Tillage Research* **79**(1), 7-31.

SOLANS, M., PELLIZA, Y. I. & TADEY, M. (2022). Inoculation with Native Actinobacteria May Improve Desert Plant Growth and Survival with Potential Use for Restoration Practices. *Microbial Ecology* **83**(2), 380-392.

STANDISH, R. J., CRAMER, V. A., HOBBS, R. J. & KOBRYN, H. T. (2006). Legacy of Land-Use Evident in Soils of Western Australia's Wheatbelt. *Plant and Soil* **280**(1), 189-207.

STANDISH, R. J. & HOBBS, R. J. (2010). Restoration of OCBILs in south-western Australia: response to Hopper. *Plant and Soil* **330**, 15-18.

STEINAUER, K., TILMAN, D., WRAGG, P. D., CESARZ, S., COWLES, J. M., PRITSCH, K., REICH, P. B., WEISSER, W. W. & EISENHAUER, N. (2015). Plant diversity effects on soil microbial functions and enzymes are stronger than warming in a grassland experiment. *Ecology* **96**(1), 99-112.

STOCK, E., STANDISH, R. J., MUÑOZ-ROJAS, M., BELL, R. W. & ERICKSON, T. E. (2020). Field-Deployed extruded seed pellets show promise for perennial grass establishment in arid zone mine rehabilitation. *Frontiers in Ecology and Evolution*, 447.

SUBEDI, Y. R., KRISTIANSEN, P. & CACHO, O. (2022). Drivers and consequences of agricultural land abandonment and its reutilisation pathways: A systematic review. *Environmental Development* **42**, 100681.

SUDING, K., HIGGS, E., PALMER, M., CALLICOTT, J. B., ANDERSON, C. B., BAKER, M., GUTRICH, J. J., HONDULA, K. L., LAFEVOR, M. C. & LARSON, B. M. (2015). Committing to ecological restoration. *Science* **348**(6235), 638-640.

SUDING, K. N., GROSS, K. L. & HOUSEMAN, G. R. (2004). Alternative states and positive feedbacks in restoration ecology. *Trends in Ecology & Evolution* **19**(1), 46-53.

SUN, R., WANG, F., HU, C. & LIU, B. (2021). Metagenomics reveals taxon-specific responses of the nitrogen-cycling microbial community to long-term nitrogen fertilization. *Soil Biology and Biochemistry* **156**, 108214.

SUN, S. & BADGLEY, B. D. (2019). Changes in microbial functional genes within the soil metagenome during forest ecosystem restoration. *Soil Biology and Biochemistry* **135**, 163-172.

SUN, S., JONES, R. B. & FODOR, A. A. (2020). Inference-based accuracy of metagenome prediction tools varies across sample types and functional categories. *Microbiome* **8**, 1-9.

SUN, S., LI, S., AVERA, B. N., STRAHM, B. D. & BADGLEY, B. D. (2017). Soil bacterial and fungal communities show distinct recovery patterns during forest ecosystem restoration. *Applied and Environmental Microbiology* **83**(14), e00966-17.

SUN, Y.-Q. & GE, Y. (2023). Relic DNA effects on the estimates of bacterial community composition and taxa dynamics in soil. *Applied Microbiology and Biotechnology* **107**(12), 4109-4117.

TEAM, R. C. (2024). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

TEDERSOO, L., BAHRAM, M., CAJTHAML, T., PÕLME, S., HIIESALU, I., ANSLAN, S., HAREND, H., BUEGGER, F., PRITSCH, K. & KORICHEVA, J. (2016). Tree diversity and species identity effects on soil fungi, protists and animals are context dependent. *The ISME Journal* **10**(2), 346-362.

TEDERSOO, L., DRENKHAN, R., ANSLAN, S., MORALES‐RODRIGUEZ, C. & CLEARY, M. (2019). High‐throughput identification and diagnostics of pathogens and pests: overview and practical recommendations. *Molecular ecology resources* **19**(1), 47-76.

THOMSEN, P. F. & WILLERSLEV, E. (2015). Environmental DNA–An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation* **183**, 4-18.

THRALL, P., MURRAY, B., WATKIN, E., WOODS, M., BAKER, K., BURDON, J. & BROCKWELL, J. (2001). Bacterial partnerships enhance the value of native legumes in rehabilitation of degraded agricultural lands. *Ecological Management and Restoration* **2**, 3.

TIBBETT, M. (2010). Large-scale mine site restoration of Australian eucalypt forests after bauxite mining: soil management and ecosystem development. In *Ecology of industrial pollution*. (pp. 309-326. Cambridge University Press UK.

TIBBETT, M. (2024). Post-mining ecosystem reconstruction. *Current Biology* **34**(9), R387-R393.

TISSERANT, E., MALBREIL, M., KUO, A., KOHLER, A., SYMEONIDI, A., BALESTRINI, R., CHARRON, P., DUENSING, N., FREI DIT FREY, N., GIANINAZZI-PEARSON, V., GILBERT, L. B., HANDA, Y., HERR, J. R., HIJRI, M., KOUL, R., KAWAGUCHI, M., KRAJINSKI, F., LAMMERS, P. J., MASCLAUX, F. G., MURAT, C., MORIN, E., NDIKUMANA, S., PAGNI, M., PETITPIERRE, D., REQUENA, N., ROSIKIEWICZ, P., RILEY, R., SAITO, K., SAN CLEMENTE, H., SHAPIRO, H., VAN TUINEN, D., BÉCARD, G., BONFANTE, P., PASZKOWSKI, U., SHACHAR-HILL, Y. Y., TUSKAN, G. A., YOUNG, J. P. W., SANDERS, I. R., HENRISSAT, B., RENSING, S. A., GRIGORIEV, I. V., CORRADI, N., ROUX, C. & MARTIN, F. (2013). Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. *Proceedings of the National Academy of Sciences* **110**(50), 20117-20122.

TOLEDO, R. M., SANTOS, R. F., BAETEN, L., PERRING, M. P. & VERHEYEN, K. (2018). Soil properties and neighbouring forest cover affect above-ground biomass and functional composition during tropical forest restoration. *Applied Vegetation Science* **21**(2), 179-189.

TREJO-AGUILAR, D., LARA-CAPISTRÁN, L., MALDONADO-MENDOZA, I. E., ZULUETA-RODRÍGUEZ, R., SANGABRIEL-CONDE, W., MANCERA-LÓPEZ, M. E., NEGRETE-YANKELEVICH, S. & BAROIS, I. (2013). Loss of arbuscular mycorrhizal fungal diversity in trap cultures during long-term subculturing. *IMA fungus* **4**(2), 161-167.

TULLOCH, A. I. T., BARNES, M. D., RINGMA, J., FULLER, R. A. & WATSON, J. E. M. (2016). Understanding the importance of small patches of habitat for conservation. *Journal of Applied Ecology* **53**(2), 418-429.

TURLEY, N. E., BELL-DERESKE, L., EVANS, S. E. & BRUDVIG, L. A. (2020). Agricultural land-use history and restoration impact soil microbial biodiversity. *Journal of Applied Ecology* **57**(5), 852-863.

UNIVERSITY, F. (2021). Deep Thought (HPC). Flinders University.

VALLIERE, J. M., D'AGUI, H. M., DIXON, K. W., NEVILL, P. G., WONG, W. S., ZHONG, H. & VENEKLAAS, E. J. (2022). Stockpiling disrupts the biological integrity of topsoil for ecological restoration. *Plant and Soil* **471**(1), 409-426.

VALLIERE, J. M., WONG, W. S., NEVILL, P. G., ZHONG, H. T. & DIXON, K. W. (2020). Preparing for the worst: Utilizing stress-tolerant soil microbial communities to aid ecological restoration in the Anthropocene. *Ecological Solutions and Evidence* **1**(2), 12.

VAN DER BIJ, A. U., WEIJTERS, M. J., BOBBINK, R., HARRIS, J. A., PAWLETT, M., RITZ, K., BENETKOVÁ, P., MORADI, J., FROUZ, J. & VAN DIGGELEN, R. (2018). Facilitating ecosystem assembly: Plant-soil interactions as a restoration tool. *Biological Conservation* **220**, 272-279.

VAN DER HEYDE, M., BUNCE, M., DIXON, K., WARDELL-JOHNSON, G., WHITE, N. & NEVILL, P. (2020). Changes in soil microbial communities in post mine ecological restoration: Implications for monitoring using high throughput DNA sequencing. *Science of The Total Environment* **749**, 142262.

VAN DER HEYDE, M., BUNCE, M. & NEVILL, P. (2022). Key factors to consider in the use of environmental DNA metabarcoding to monitor terrestrial ecological restoration. *Science of The Total Environment*, 157617.

VAN DER HEYDE, M., OHSOWSKI, B., ABBOTT, L. K. & HART, M. (2017). Arbuscular mycorrhizal fungus responses to disturbance are context-dependent. *Mycorrhiza* **27**(5), 431-440.

VAN DER PUTTEN, W. H., BRADFORD, M. A., PERNILLA BRINKMAN, E., VAN DE VOORDE, T. F. J. & VEEN, G. F. (2016). Where, when and how plant–soil feedback matters in a changing world. *Functional Ecology* **30**(7), 1109-1121.

VAN GOOL, D., STUART-STREET, A. & TILLE, P. (2018). Distribution of classified soils in south-west Western Australia.

VARTOUKIAN, S. R., PALMER, R. M. & WADE, W. G. (2010). Strategies for culture of 'unculturable' bacteria. *FEMS Microbiology Letters* **309**(1), 1-7.

VASSILEV, N., VASSILEVA, M., MARTOS, V., GARCIA DEL MORAL, L. F., KOWALSKA, J., TYLKOWSKI, B. & MALUSÁ, E. (2020). Formulation of Microbial Inoculants by Encapsulation in Natural Polysaccharides: Focus on Beneficial Properties of Carrier Additives and Derivatives. *Frontiers in Plant Science* **11**.

VITOUSEK, P. M., MENGE, D. N. L., REED, S. C. & CLEVELAND, C. C. (2013). Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. *Philosophical Transactions of the Royal Society B: Biological Sciences* **368**(1621), 20130119.

WALKER, L. R., WARDLE, D. A., BARDGETT, R. D. & CLARKSON, B. D. (2010). The use of chronosequences in studies of ecological succession and soil development. *Journal of ecology* **98**(4), 725-736.

Walters, K. E., Capocchi, J. K., Albright, M. B. N., Hao, Z., Brodie, E. L. & Martiny, J. B. H. (2022). Routes and rates of bacterial dispersal impact surface soil microbiome composition and functioning. *The ISME journal* **16**(10), 2295-2304.

Waltham, N. J., Elliott, M., Lee, S. Y., Lovelock, C., Duarte, C. M., Buelow, C., Simenstad, C., Nagelkerken, I., Claassens, L., Wen, C. K. C., Barletta, M., Connolly, R. M., Gillies, C., Mitsch, W. J., Ogburn, M. B., Purandare, J., Possingham, H. & Sheaves, M. (2020). UN Decade on Ecosystem Restoration 2021–2030—What Chance for Success in Restoring Coastal Ecosystems? *Frontiers in Marine Science* **7**.

Wang, C., Ma, L., Zuo, X., Ye, X., Wang, R., Huang, Z., Liu, G. & Cornelissen, J. H. C. (2022). Plant diversity has stronger linkage with soil fungal diversity than with bacterial diversity across grasslands of northern China. *Global ecology and Biogeography* **31**(5), 886-900.

Wang, Q., Garrity, G. M., Tiedje, J. M. & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73**(16), 5261-5267.

Wang, Y., Bing, H., Moorhead, D. L., Hou, E., Wu, Y., Wang, J., Duan, C., Cui, Q., Zhang, Z., Zhu, H., Qiu, T., Dai, Z., Tan, W., Huang, M., Lambers, H., Reich, P. B. & Fang, L. (2024). Bacterial Community Structure Modulates Soil Phosphorus Turnover at Early Stages of Primary Succession. *Global Biogeochemical Cycles* **38**(10), e2024GB008174.

Waterhouse, B. R., Adair, K. L., Boyer, S. & Wratten, S. D. (2014). Advanced mine restoration protocols facilitate early recovery of soil microbial biomass, activity and functional diversity. *Basic and Applied Ecology* **15**(7), 599-606.

Watson, C. D., Gardner, M. G., Hodgson, R. J., Liddicoat, C., Peddle, S. D. & Breed, M. F. (2022). Global meta-analysis shows progress towards recovery of soil microbiota following revegetation. *Biological Conservation* **272**, 109592.

Weber, B., Belnap, J., Büdel, B., Antoninka, A. J., Barger, N. N., Chaudhary, V. B., Darrouzet-Nardi, A., Eldridge, D. J., Faist, A. M., Ferrenberg, S., Havrilla, C. A., Huber-Sannwald, E., Malam Issa, O., Maestre, F. T., Reed, S. C., Rodriguez-Caballero, E., Tucker, C., Young, K. E., Zhang, Y., Zhao, Y., Zhou, X. & Bowker, M. A. (2022). What is a biocrust? A refined, contemporary definition for a broadening research community. *Biological Reviews* **97**(5), 1768-1785.

Weinstein, M. M., Prem, A., Jin, M., Tang, S. & Bhasin, J. M. (2019). FIGARO: An efficient and objective tool for optimizing microbiome rRNA gene trimming parameters. *bioRxiv*, 610394.

Wemheuer, F., Taylor, J. A., Daniel, R., Johnston, E., Meinicke, P., Thomas, T. & Wemheuer, B. (2020). Tax4Fun2: prediction of habitat-specific functional profiles and functional redundancy based on 16S rRNA gene sequences. *Environmental Microbiome* **15**(1), 11.

WEST, J. R. & WHITMAN, T. (2022). Disturbance by soil mixing decreases microbial richness and supports homogenizing community assembly processes. *FEMS Microbiology Ecology* **98**(9).

WHEELER, B., TORCHIANO, M. & TORCHIANO, M. M. (2016). Package 'lmPerm'. *R package version*, 1-1.

WHITE, P. S. & WALKER, J. L. (1997). Approximating Nature's Variation: Selecting and Using Reference Information in Restoration Ecology. *Restoration ecology* **5**(4), 338-349.

WILLIS, A., RODRIGUES, B. F. & HARRIS, P. J. C. (2013). The Ecology of Arbuscular Mycorrhizal Fungi. *Critical Reviews in Plant Sciences* **32**(1), 1-20.

WINTLE, B. A., KUJALA, H., WHITEHEAD, A., CAMERON, A., VELOZ, S., KUKKALA, A., MOILANEN, A., GORDON, A., LENTINI, P. E., CADENHEAD, N. C. R. & BEKESSY, S. A. (2019). Global synthesis of conservation studies reveals the importance of small habitat patches for biodiversity. *Proceedings of the National Academy of Sciences* **116**(3), 909-914.

WOOD, D. E., LU, J. & LANGMEAD, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome Biology* **20**(1), 257.

WORTLEY, L., HERO, J. M. & HOWES, M. (2013). Evaluating ecological restoration success: a review of the literature. *Restoration Ecology* **21**(5), 537-543.

WUBS, E. J., VAN DER PUTTEN, W. H., BOSCH, M. & BEZEMER, T. M. (2016a). Soil inoculation steers restoration of terrestrial ecosystems. *Nature Plants* **2**(8), 1-5.

WUBS, E. R. J., VAN DER PUTTEN, W. H., BOSCH, M. & BEZEMER, T. M. (2016b). Soil inoculation steers restoration of terrestrial ecosystems. *Nature Plants* **2**(8), 5.

WUBS, E. R. J., VAN DER PUTTEN, W. H., MORTIMER, S. R., KORTHALS, G. W., DUYTS, H., WAGENAAR, R. & BEZEMER, T. M. (2019a). Single introductions of soil biota and plants generate long-term legacies in soil and plant community assembly. *Ecology Letters* **22**(7), 1145-1151.

WUBS, E. R. J., VAN HEUSDEN, T., MELCHERS, P. D. & BEZEMER, T. M. (2019b). Soil Inoculation Steers Plant-Soil Feedback, Suppressing Ruderal Plant Species. *Frontiers in Ecology and Evolution* **7**(451).

YAN, D., BISSETT, A., GELLIE, N., MILLS, J. G., LOWE, A. J. & BREED, M. F. (2019). Soil bacterial community differences along a coastal restoration chronosequence. *Plant Ecology*, 1-17.

YAN-GUI, S., XIN-RONG, L., YING-WU, C., ZHI-SHAN, Z. & YAN, L. (2013). CARBON FIXATION OF CYANOBACTERIAL–ALGAL CRUSTS AFTER DESERT FIXATION AND ITS IMPLICATION TO SOIL ORGANIC CARBON ACCUMULATION IN DESERT. *Land Degradation & Development* **24**(4), 342-349.

YANG, G., RYO, M., ROY, J., HEMPEL, S. & RILLIG, M. C. (2021). Plant and soil biodiversity have non‐substitutable stabilising effects on biomass production. *Ecology Letters* **24**(8), 1582-1593.

YAO, Q., LI, Z., SONG, Y., WRIGHT, S. J., GUO, X., TRINGE, S. G., TFAILY, M. M., PAŠA-TOLIĆ, L., HAZEN, T. C., TURNER, B. L., MAYES, M. A. & PAN, C. (2018). Community proteogenomics reveals the systemic impact of phosphorus availability on microbial functions in tropical soil. *Nature Ecology & Evolution* **2**(3), 499-509.

ZHU, C., FRIMAN, V.-P., LI, L., XU, Q., GUO, J., GUO, S., SHEN, Q. & LING, N. (2022). Meta-analysis of diazotrophic signatures across terrestrial ecosystems at the continental scale. *Environmental Microbiology* **24**(4), 2013-2028.

ZINNEN, J., BROADHURST, L. M., GIBSON-ROY, P., JONES, T. A. & MATTHEWS, J. W. (2021). Seed production areas are crucial to conservation outcomes: benefits and risks of an emerging restoration tool. *Biodiversity and Conservation* **30**(5), 1233-1256.

ZVINAVASHE, A. T., LIM, E., SUN, H. & MARELLI, B. (2019). A bioinspired approach to engineer seed microenvironment to boost germination and mitigate soil salinity. *Proceedings of the National Academy of Sciences* **116**(51), 25555-25561.

# Appendices

## Appendix 1:

## Supplementary material from Chapter 2, *Agricultural land-use legacies affect soil bacterial communities following restoration in a global biodiversity hotspot*

Shawn D. Peddle[1], Christian Cando-Dumancela[1], Siegfried L. Krauss[2,3], Craig Liddicoat[1], Angela Sanders[4], Martin F. Breed[1]

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[2]*The University of Western Australia, Crawley, WA, 6009, Australia.*

[3]*Kings Park Science, Department of Biodiversity, Conservation and Attractions, Perth, WA, 6005, Australia.*

[4]*Bush Heritage Australia, Albany, WA, 6330, Australia.*

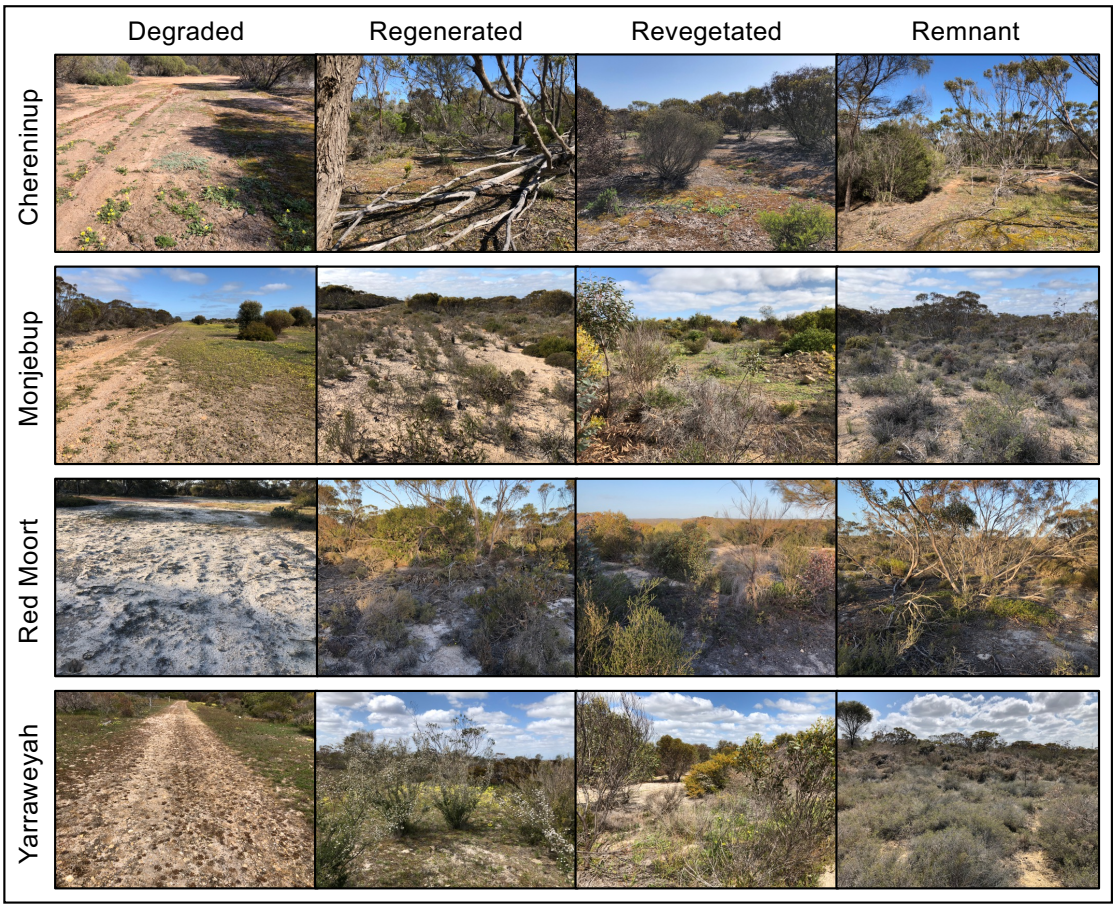



***Figure S1.*** *Photographs from soil sampling sites showing the four land conditions (Degraded, Regenerated, Revegetated, and Remnant) at four sites (Chereninup Reserve, Monjebup Reserve, Red Moort Reserve, and Yarraweyah Falls).*

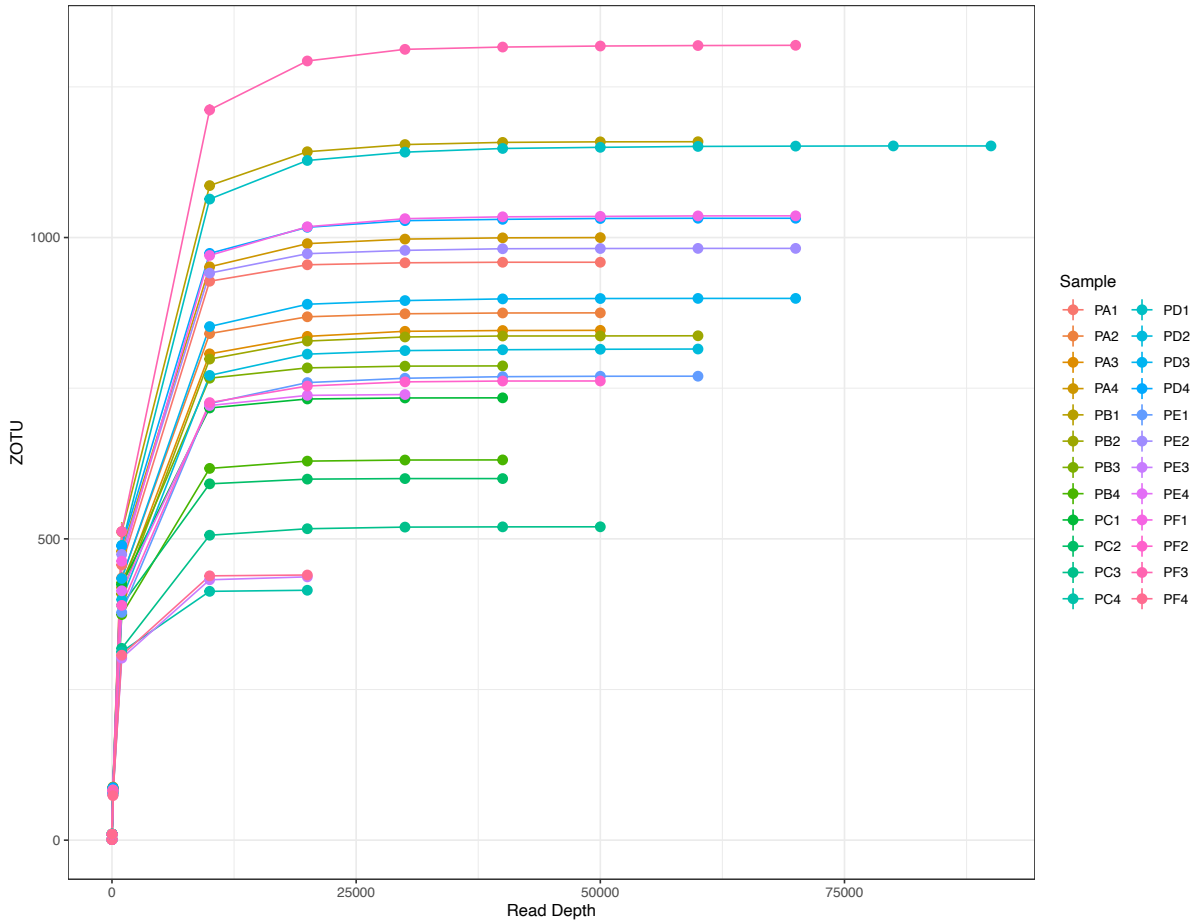



***Figure S2.*** *Zero-radius operational taxonomic unit (zOTU) accumulation curve comparing observed zOTU richness and sample sequence read depth to assess if sample zOTU richness was adequately represented by read depth and to determine an appropriate read depth for rarefaction to ensure unbiased comparisons across samples. All samples were rarefied to 22,427 reads to match the sample with the lowest read depth.*

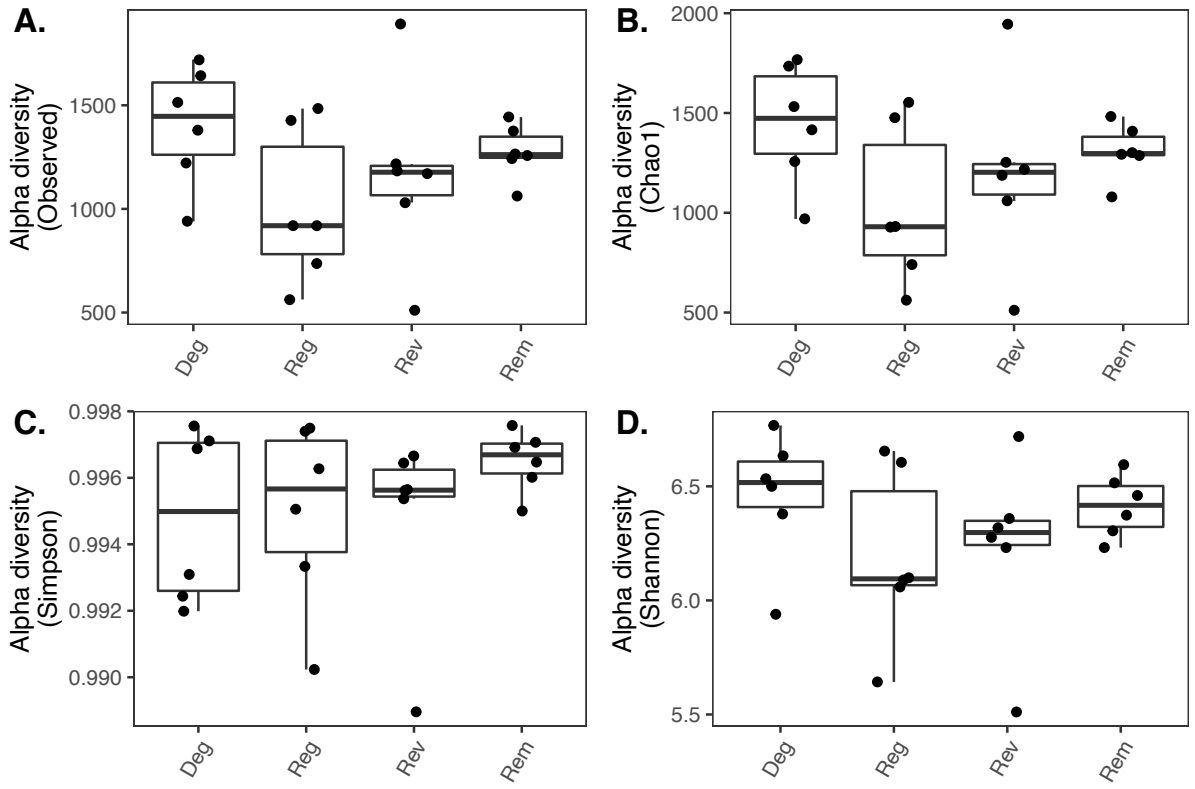



***Figure S3.*** *Boxplots indicating soil bacterial richness and alpha diversity values from soil samples collected from four land conditions (Degraded, Regenerated, Revegetated, and Remnant) at six restoration sites (Chereninup Reserve, Monjebup Reserve, Red Moort Reserve, and Yarraweyah Falls) in southwest Western Australia. A) shows Observed Richness, B) Chao1 Richness, C) Simpson's diversity, and D) Shannon diversity. No measured richness or alpha diversity metric differed by land condition (ANOVA $p > 0.05$ in all cases).*

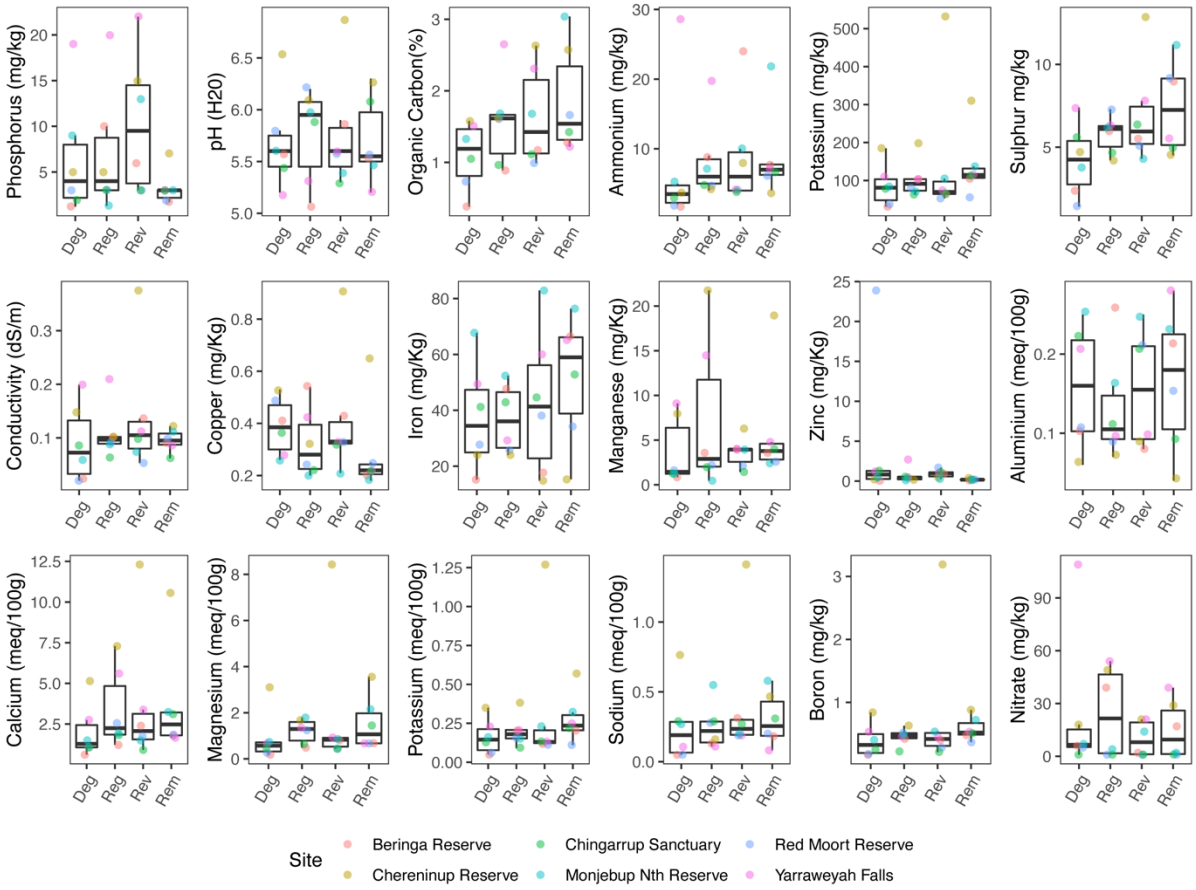



***Figure S4.*** *Boxplots of measured soil chemical variables across the four land conditions. No soil chemical property significantly varied ($p > 0.05$ for all) by land condition. Colour indicates site.*

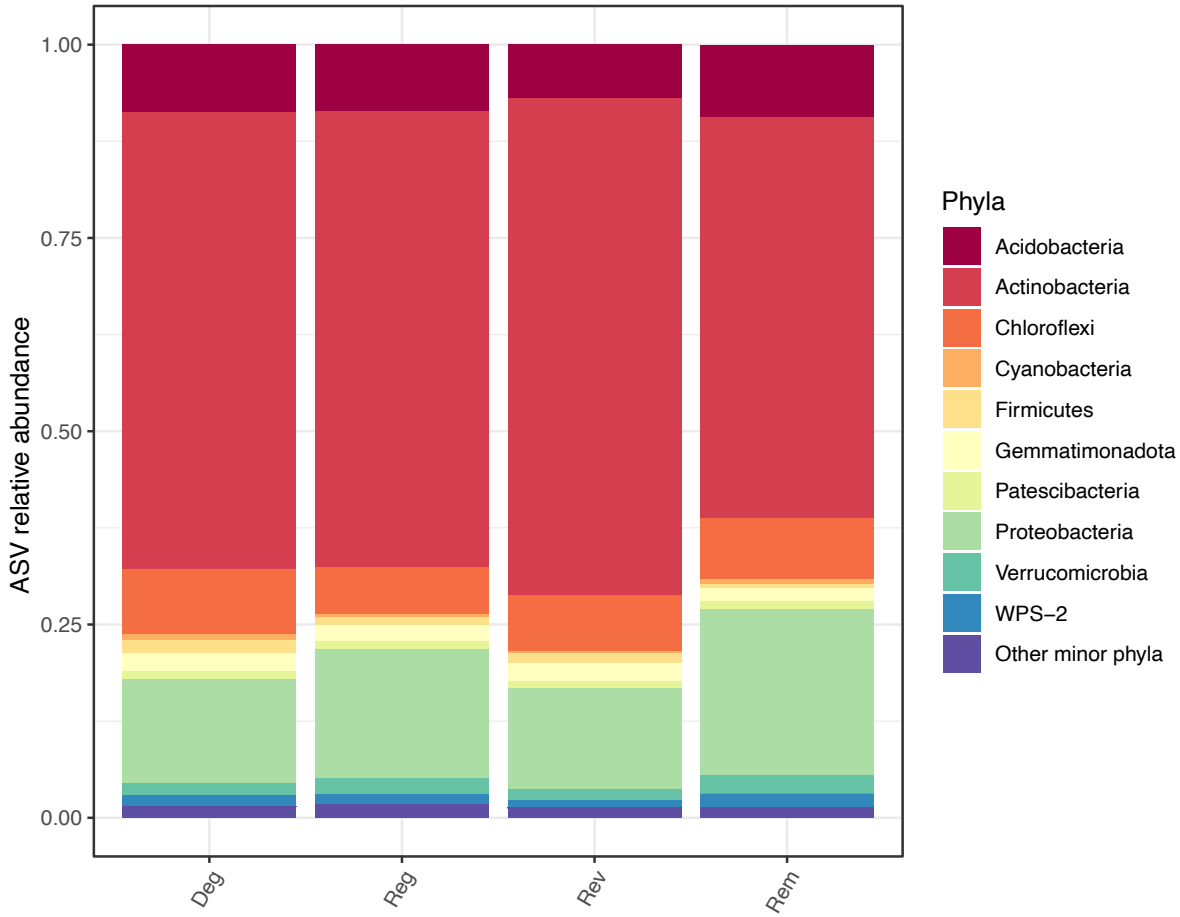



***Figure S5.*** *Rarefied relative abundance of the ten most abundant bacterial phyla across the four land conditions. These ten phyla represent 98.56% of total bacterial abundance and the remaining 1.44% are grouped as other minor phyla. Of the ten most abundant phyla, only Proteobacteria differed (permuted ANOVA, p=0.001) across land condition.*

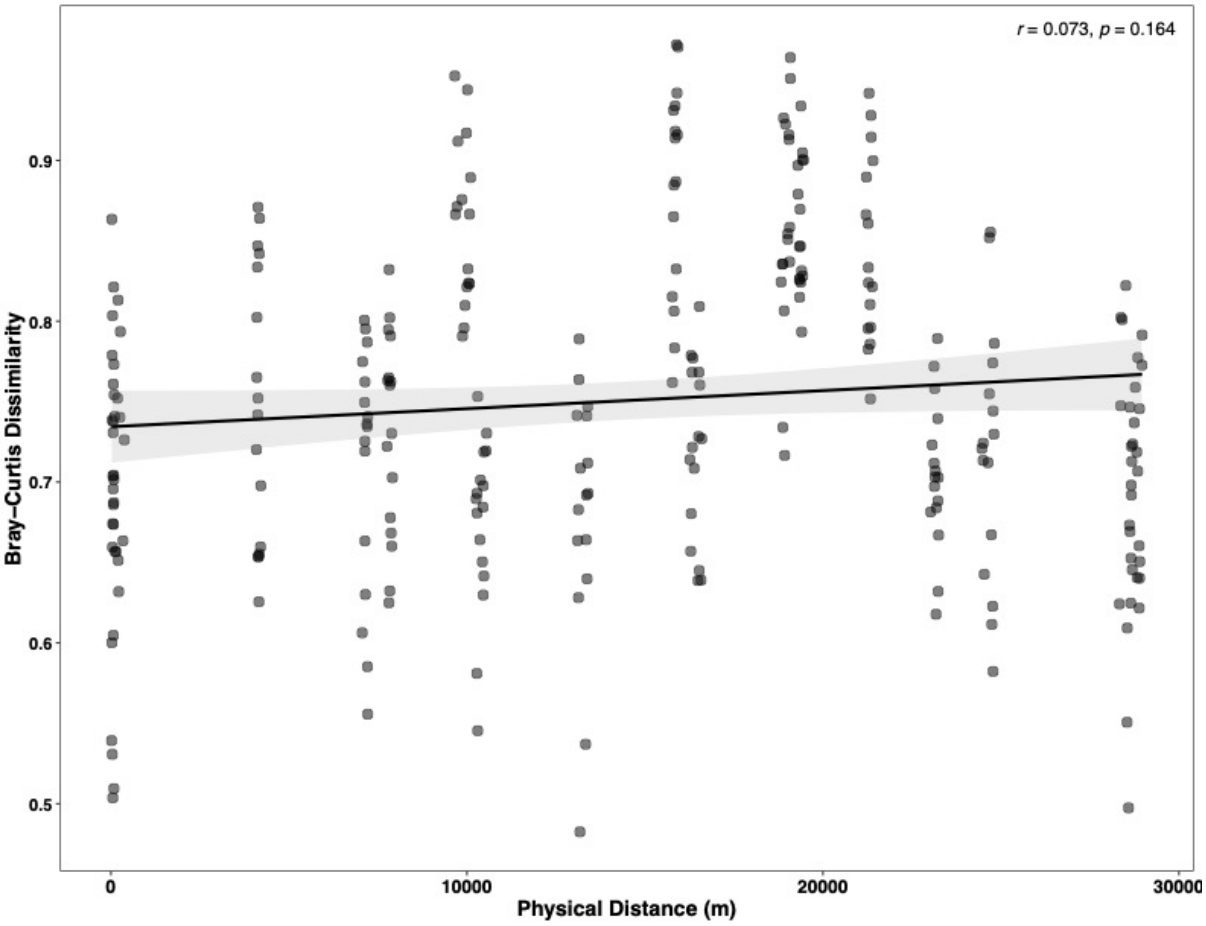



***Figure S6.*** *A scatterplot of the association between geographic distance between each sample (Haversine distance) and Bray-Curtis distance representing bacterial community composition indicating no significant spatial autocorrelation (Mantel; r = 0.723, p = 0.164).*

# Appendix 2: Supplementary material from Chapter 3, *Soil microbial functions associate with persistent agricultural legacies and indicate an alternative stable state following restoration plantings in a global biodiversity hotspot*

Shawn D. Peddle[1], Christian Cando-Dumancela[1], Sofie Costin[1], Tarryn Davies[1], Michael P. Doane[1], Robert A. Edwards[1], Riley J. Hodgson[1], Siegfried L. Krauss[2,3], Craig Liddicoat[1], Martin F. Breed[1]

[1]College of Science and Engineering, Flinders University, Bedford Park, SA, Australia. [2]The University of Western Australia, Crawley, WA, Australia. [3]Kings Park Science, Department of Biodiversity, Conservation and Attractions, Perth, WA, Australia.

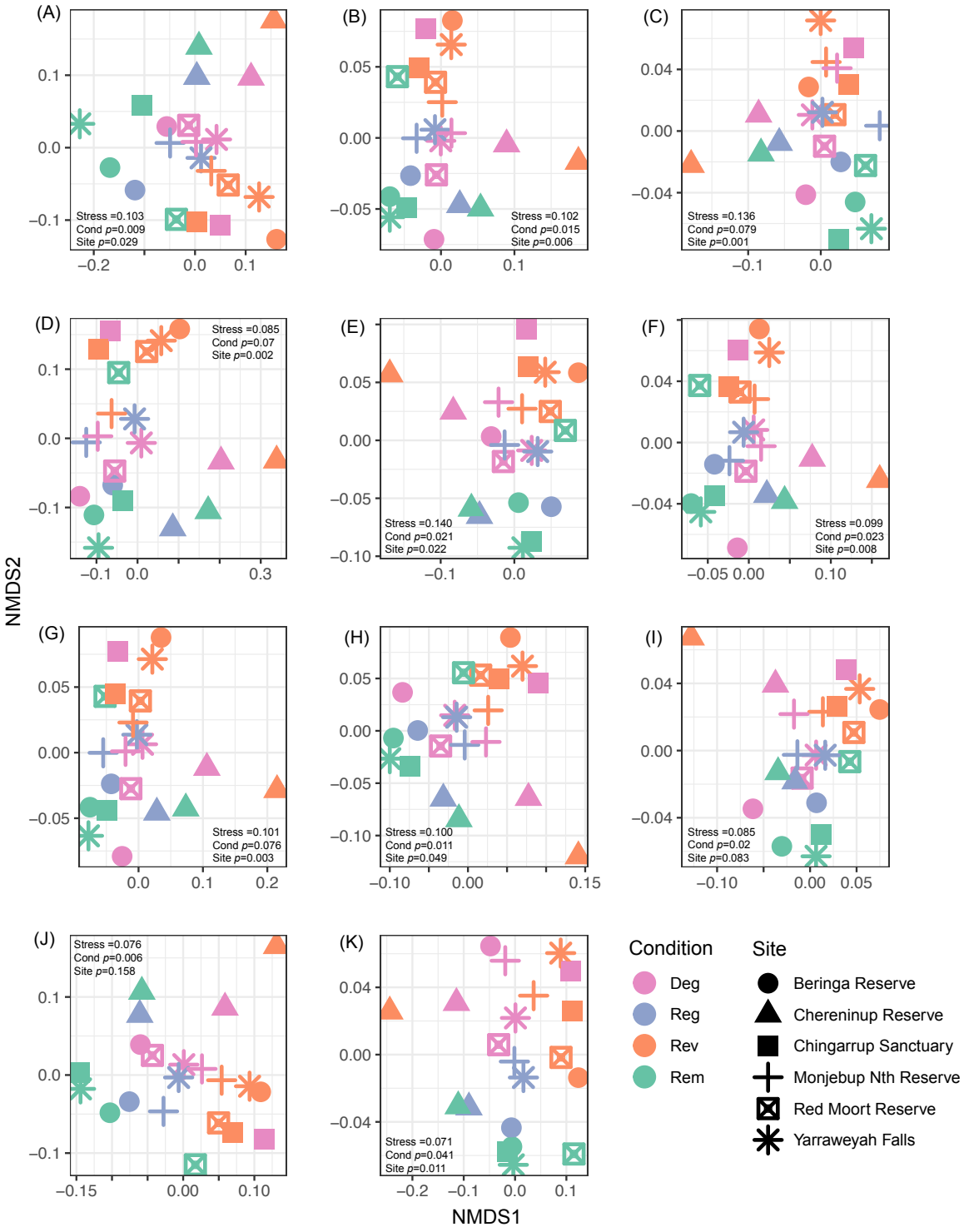
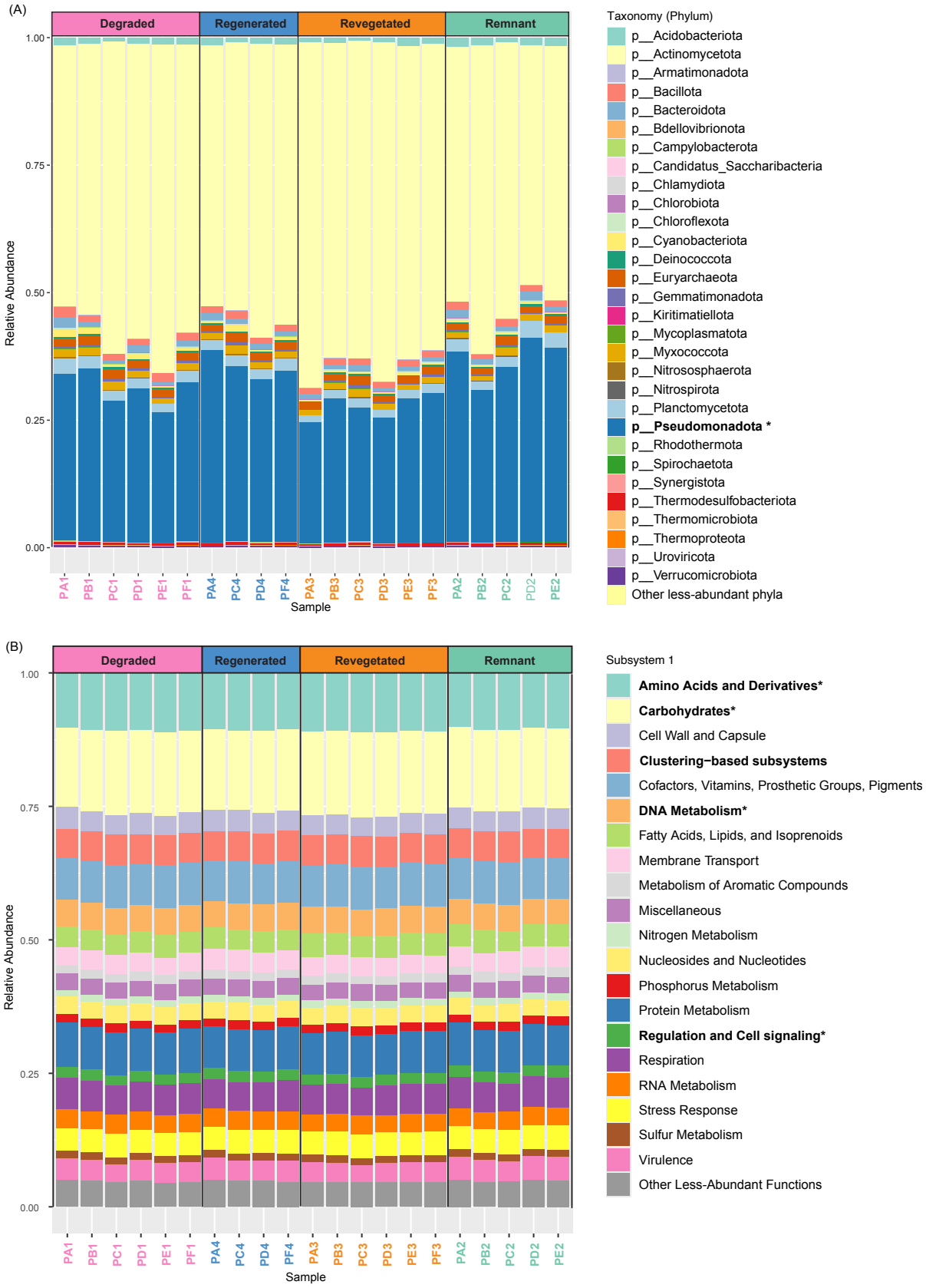



**Figure S1** Ordinations of all assessed groups. (A) taxonomy, (B) all functions, (C) phosphorus metabolism, (D) iron acquisition and metabolism, (E) sulphur acquisition and metabolism, (F) amino acids and derivatives, (G) carbohydrates, (H) clustering-based subsystems, (I) DNA metabolism, (J) regulation and cell signalling, and (K)

nitrogen metabolism. Ordinations indicate compositions of either microbial communities (taxonomy) or functions (all other) across the four land conditions and six sites.


(A)
Degraded
Regenerated
Revegetated
Remnant
Relative Abundance
1.00
0.75
0.50
0.25
0.00
PA1 PB1 PC1 PD1 PE1 PF1 PA4 PC4 PD4 PF4 PA3 PB3 PC3 PD3 PE3 PF3 PA2 PB2 PC2 PD2 PE2
Sample
Taxonomy (Phylum)
p__Acidobacteriota
p__Actinomycetota
p__Armatimonadota
p__Bacillota
p__Bacteroidota
p__Bdellovibrionota
p__Campylobacterota
p__Candidatus_Saccharibacteria
p__Chlamydiota
p__Chlorobiota
p__Chloroflexota
p__Cyanobacteriota
p__Deinococcota
p__Euryarchaeota
p__Gemmatimonadota
p__Kiritimatiellota
p__Mycoplasmatota
p__Myxococcota
p__Nitrososphaerota
p__Nitrospirota
p__Planctomycetota
p__Pseudomonadota *
p__Rhodothermota
p__Spirochaetota
p__Synergistota
p__Thermodesulfobacteriota
p__Thermomicrobiota
p__Thermoproteota
p__Uroviricota
p__Verrucomicrobiota
Other less-abundant phyla


(B)
Degraded
Regenerated
Revegetated
Remnant
Relative Abundance
1.00
0.75
0.50
0.25
0.00
PA1 PB1 PC1 PD1 PE1 PF1 PA4 PC4 PD4 PF4 PA3 PB3 PC3 PD3 PE3 PF3 PA2 PB2 PC2 PD2 PE2
Sample
Subsystem 1
Amino Acids and Derivatives*
Carbohydrates*
Cell Wall and Capsule
Clustering−based subsystems
Cofactors, Vitamins, Prosthetic Groups, Pigments
DNA Metabolism*
Fatty Acids, Lipids, and Isoprenoids
Membrane Transport
Metabolism of Aromatic Compounds
Miscellaneous
Nitrogen Metabolism
Nucleosides and Nucleotides
Phosphorus Metabolism
Protein Metabolism
Regulation and Cell signaling*
Respiration
RNA Metabolism
Stress Response
Sulfur Metabolism
Virulence
Other Less-Abundant Functions

4535

**Figure S2** Relative abundance stacked bar plots of (A) Phylum level (taxonomic) relative abundance and (B) subsystem 1 (function) relative abundance. Bolded labels indicate levels that significantly differ (permuted ANOVA, $p < 0.05$) across land condition.

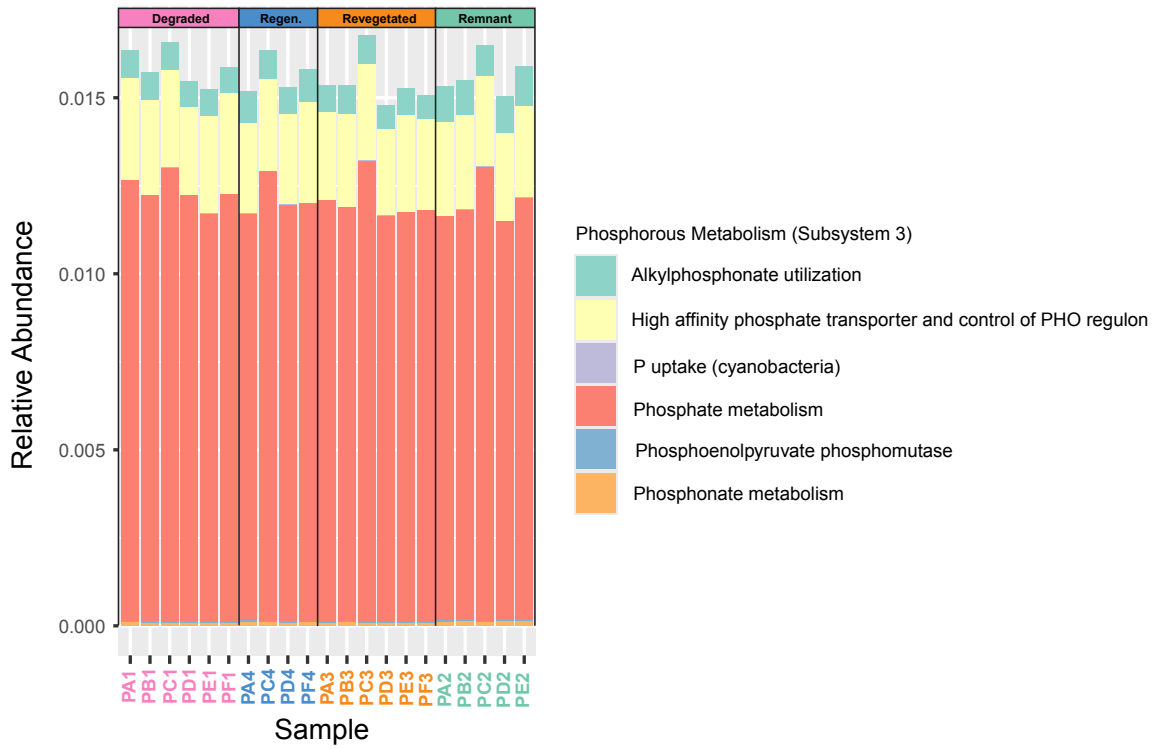



**Figure S3** Relative abundance stacked bar plot of phosphorus metabolism functions at subsystem 3. No subsystem 3 phosphorus metabolism functional groups differed (permuted ANOVA, $p > 0.05$) across land condition.

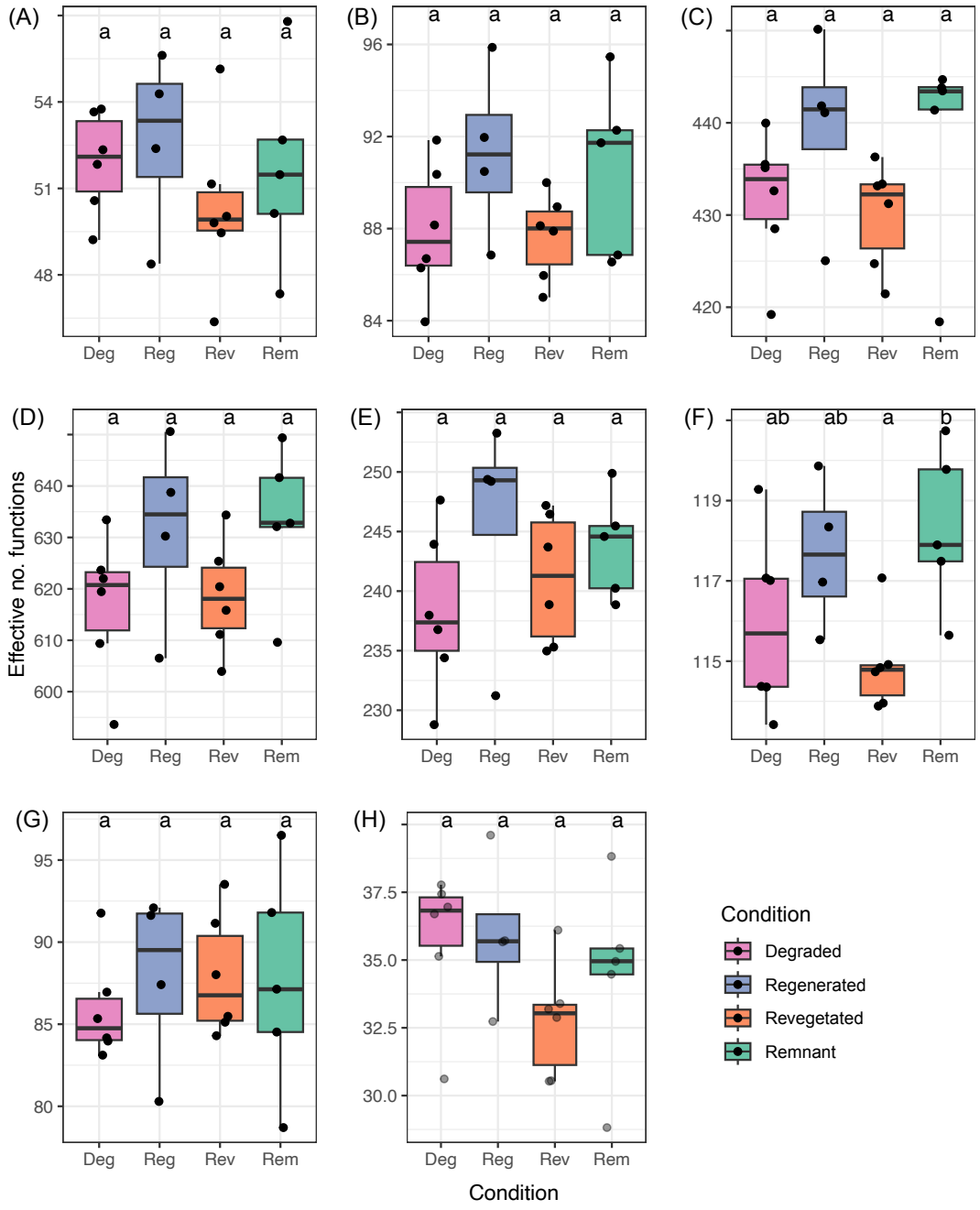



**Figure S4** Effective number of functions (alpha diversity) boxplots for all other assessed subsystem 1 levels: (A) Iron acquisition and metabolism, (B) sulphur acquisition and metabolism, (C) amino acids and derivatives, (D) carbohydrates, (E) clustering-based subsystems, (F) DNA metabolism, (G) regulation and cell signalling, and (H) nitrogen metabolism. Groups not sharing a letter are significantly different (ANOVA, $p < 0.05$).

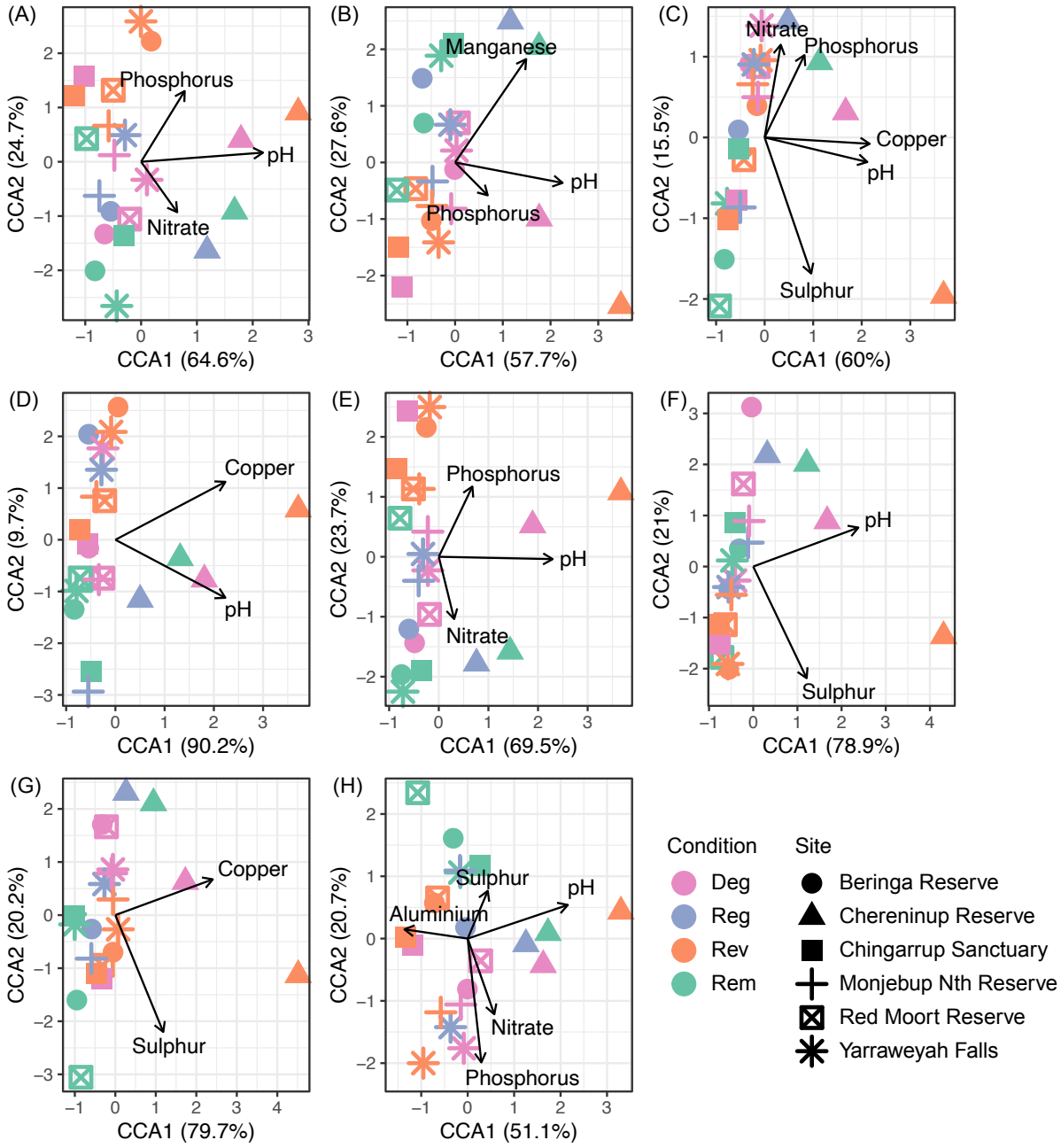



**Figure S5** Constrained correspondence analysis (CCA) ordinations indicating associations between functional compositions and model-selected soil physicochemical properties for all other assessed subsystem 1 levels: (A) Iron acquisition and metabolism, (B) sulphur acquisition and metabolism, (C) amino acids and derivatives, (D) carbohydrates, (E) clustering-based subsystems, (F) DNA metabolism, (G) regulation and cell signalling, and (H) nitrogen metabolism.

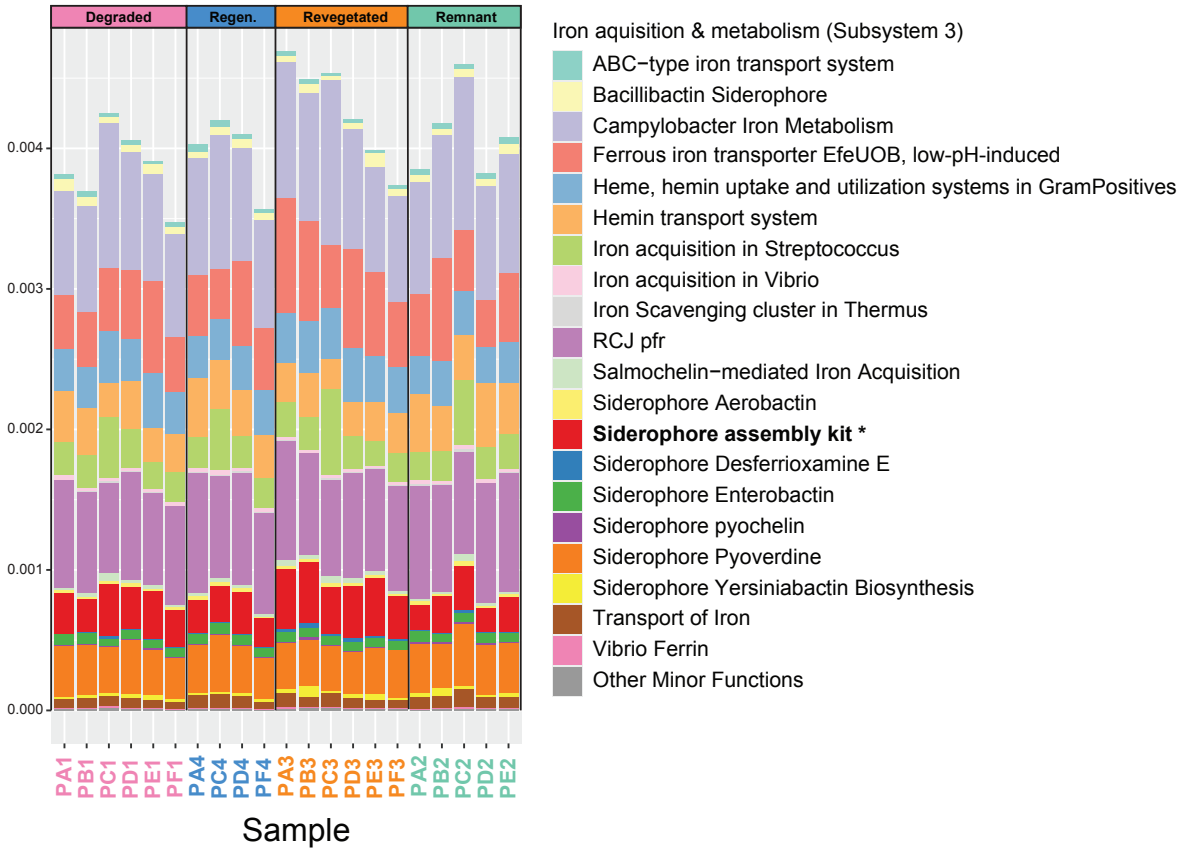



**Figure S6** Relative abundance staked bar plot of Iron acquisition and metabolism functions at subsystem 3. Bolded functions significantly differ (permuted ANOVA, $p < 0.05$) across land condition.

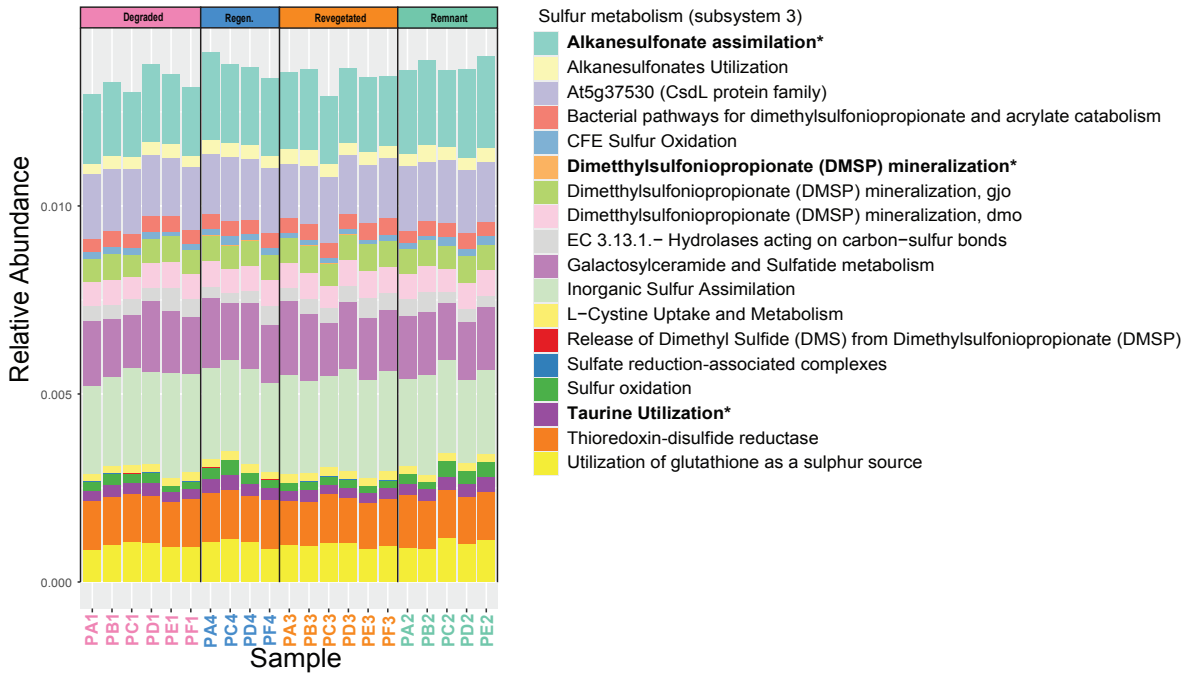



**Figure S7** Relative abundance staked bar plot of sulphur metabolism functions at subsystem 3. Bolded functions significantly differ (permuted ANOVA, $p < 0.05$) across land condition.

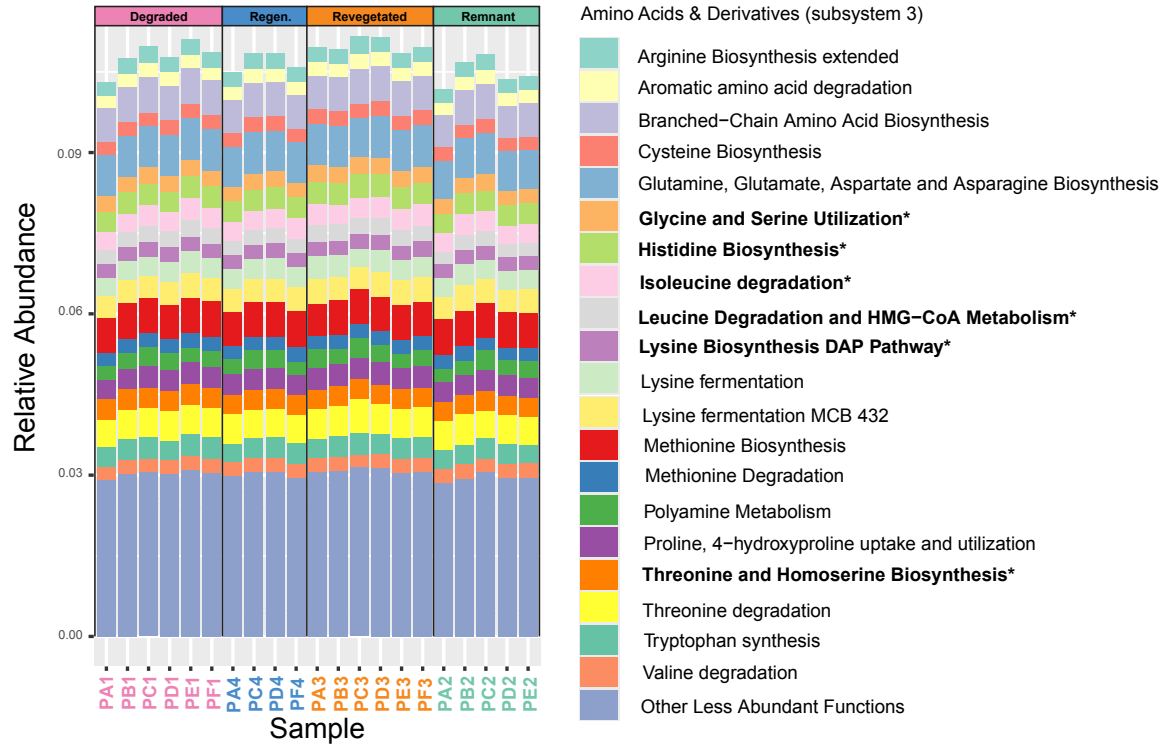


**Figure S8** Relative abundance staked bar plot of amino acids and derivatives functions at subsystem 3. Bolded functions significantly differ (permuted ANOVA, $p < 0.05$) across land condition.

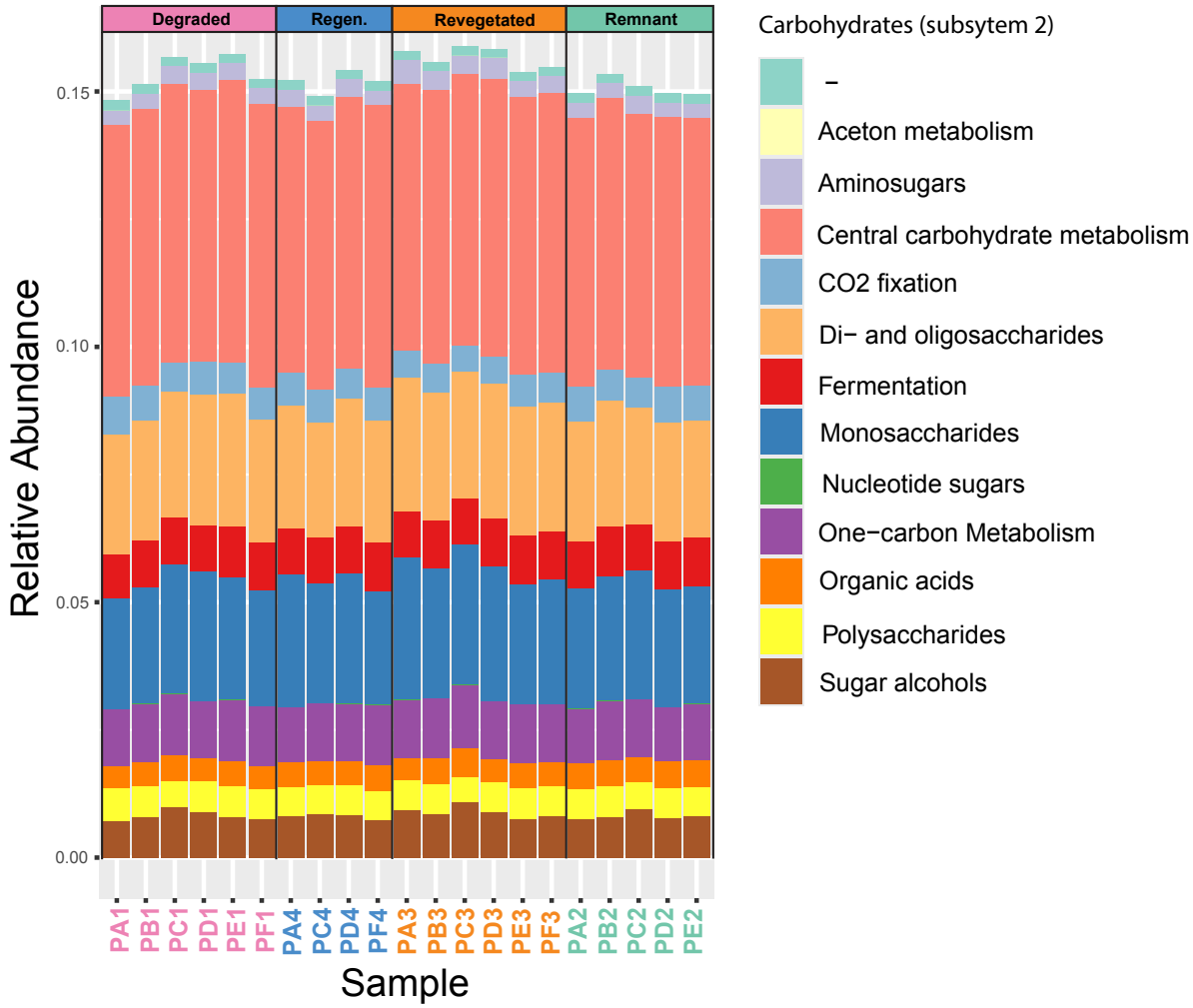



**Figure S9** Relative abundance staked bar plot of carbohydrates functions at subsystem 2. No functions significantly differed (permuted ANOVA, $p > 0.05$) across land condition.

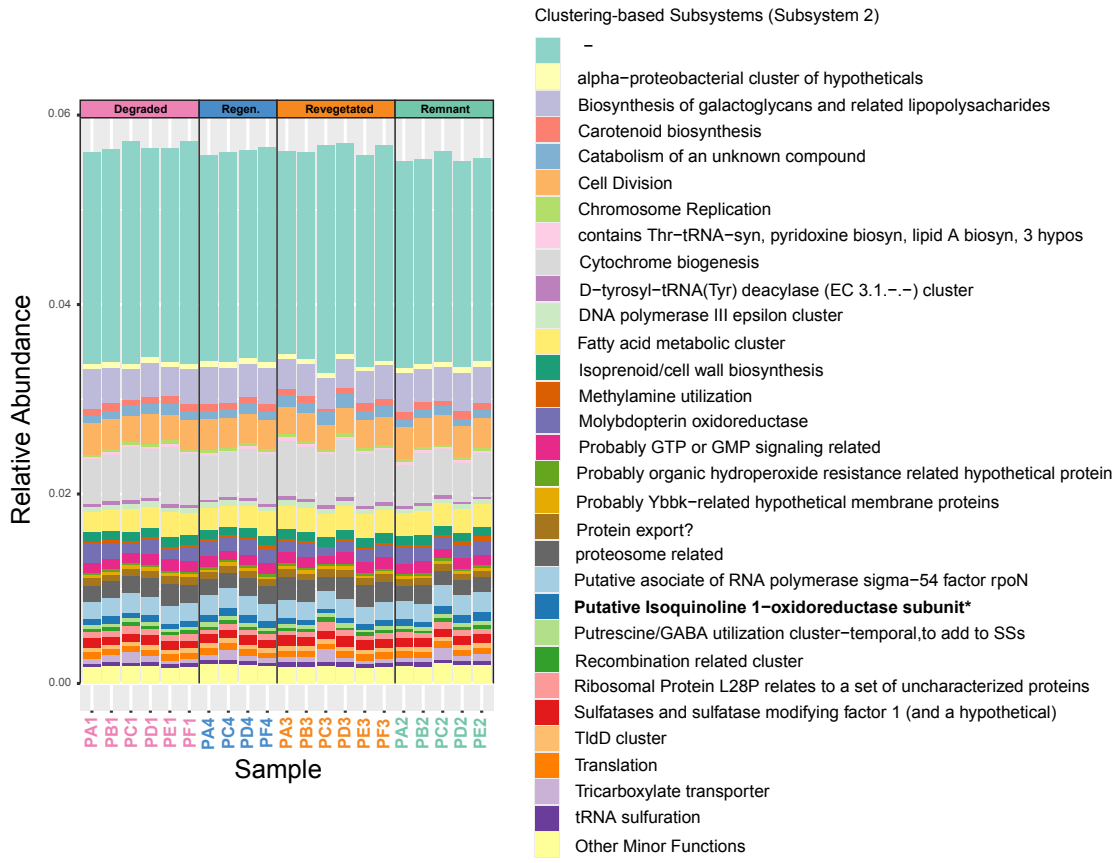



**Figure S10** Relative abundance staked bar plot of clustering-based subsystems functions at subsystem 2. Bolded functions significantly differ (permuted ANOVA, $p < 0.05$) across land condition.

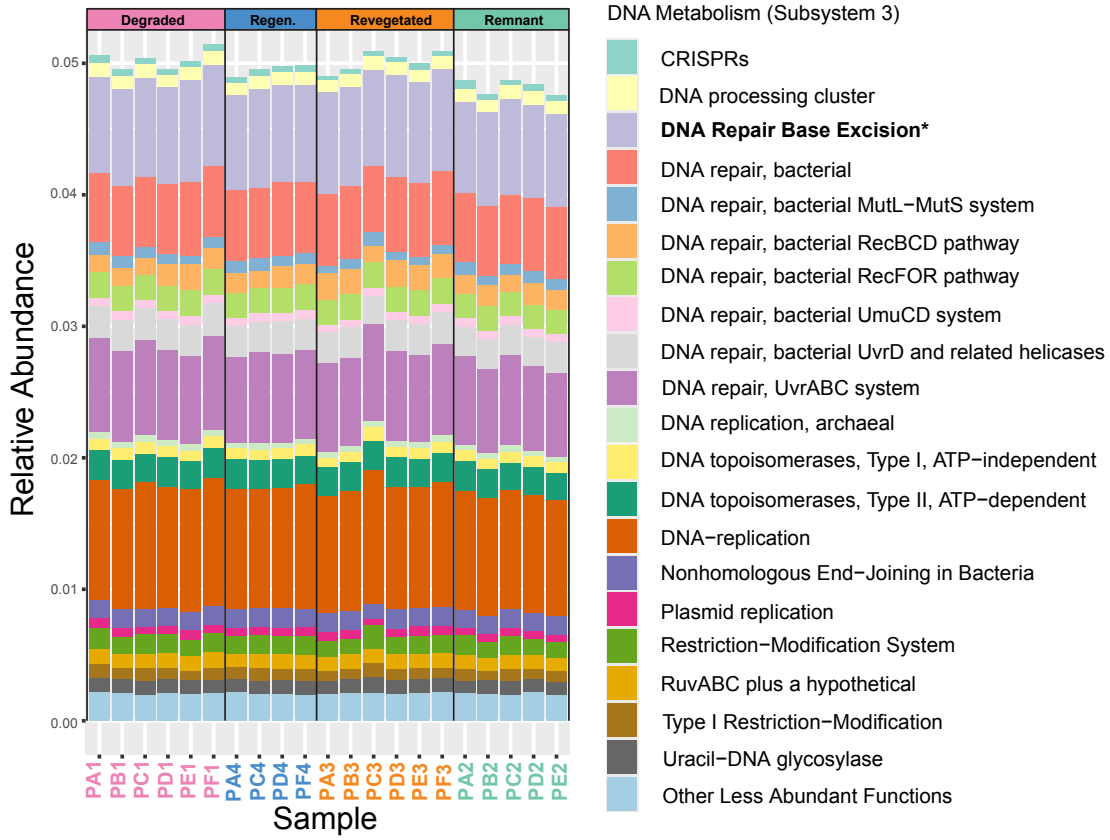



**Figure S11** Relative abundance staked bar plot of DNA metabolism functions at subsystem 3. Bolded functions significantly differ (permuted ANOVA, $p < 0.05$) across land condition.

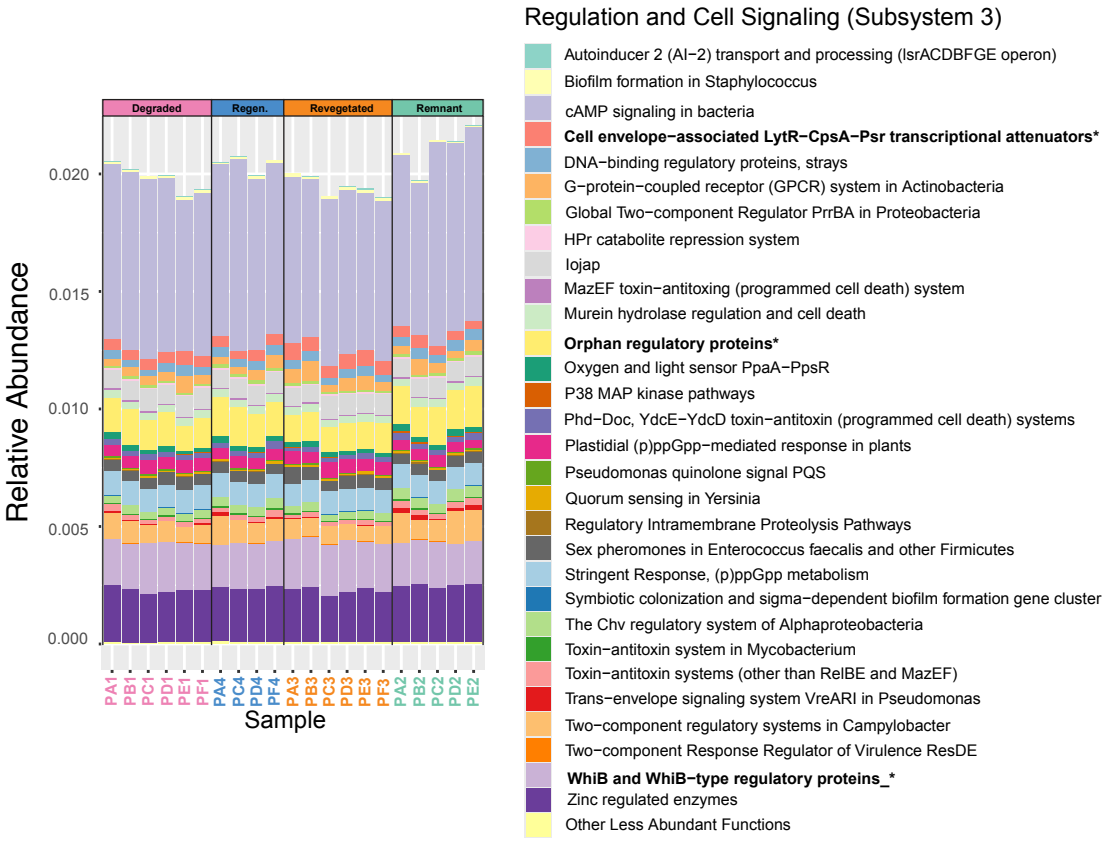



**Figure S12** Relative abundance staked bar plot of regulation and cell signalling functions at subsystem 3. Bolded functions significantly differ (permuted ANOVA, $p < 0.05$) across land condition.

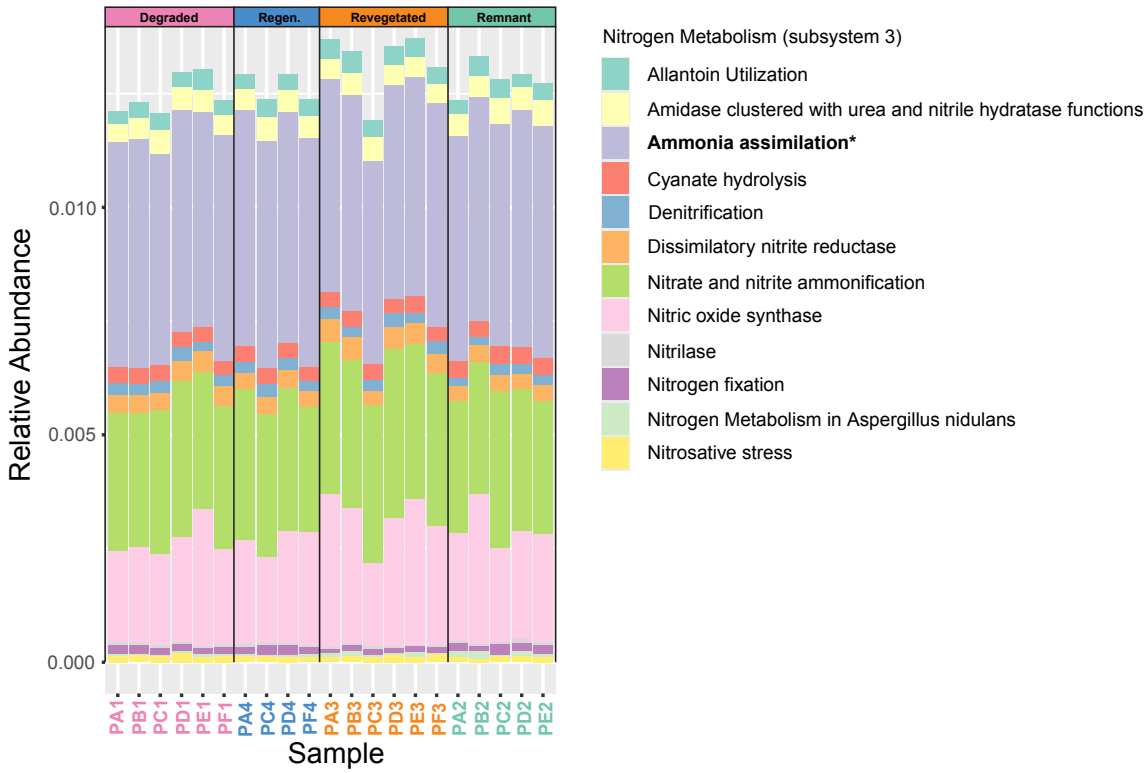



**Figure S13** Relative abundance staked bar plot of nitrogen metabolism functions at subsystem 3. Bolded functions significantly differ (permuted ANOVA, $p < 0.05$) across land condition.

**Table S1** Summary table of read depth (mean ± SD) and numbers of species and functions (mean ± SD) for each land condition. Taxonomy refers to the cleaned (Eukaryotes and taxa not assigned at the phylum level removed) taxonomic dataset and functions refers to the full non-normalised functions dataset. Number of species is at the species level and functions is at the functional process subsystem level.

| Land Condition | Taxonomy Read Depth (mean ± SD) | Functions Read Depth (mean ± SD) | Number of Species (mean ± SD) | Number of Functions (mean ± SD) |
|---|---|---|---|---|
| Degraded | 2,782,645.67 ± 1,046,248.49 | 3,218,295.33 ± 1,104,679.42 | 10,294.50 ± 336.68 | 23,791.17 ± 1,123.18 |
| Regenerated | 3,249,465.25 ± 664,193.01 | 3,772,910.00 ± 606,873.18 | 10,433.50 ± 253.24 | 24527.50 ± 774.98 |
| Revegetated | 3,482,459.50 ± 428,974.71 | 3,151,039.40 ± 1,290,283.37 | 10,388.83 ± 174.74 | 24320.83 ± 479.92 |
| Remnant | 2,751,344.60 ± 1,229,966.92 | 3,750,099.67 ± 511,453.73 | 10,278.00 ± 362.56 | 23533.40 ± 1641.90 |

**Table S2** Table of mean (± S.D.) effective number of species or functions (alpha diversity) in each land condition for each analysed group (taxonomy, all functions, or subsystem 1 functional level). Land condition levels that do not share a subscript letter indicate significant (ANOVA, $p < 0.05$) differences between land conditions within the group.

| Group | Land Condition | Effective no. species/functions (mean ± S.D.) |
|---|---|---|
| Taxonomy | Degraded | $2500.00 \pm 265.11_{a}$ |
| | Regenerated | $2450.89 \pm 95.28_{a}$ |
| | Revegetated | $2196.32 \pm 252.89_{a}$ |
| | Remnant | $2419.69 \pm 102.89_{a}$ |
| All functions | Degraded | $3622.64 \pm 63.01_{a}$ |
| | Regenerated | $3688.24 \pm 82.44_{a}$ |
| | Revegetated | $3639.05 \pm 44.81_{a}$ |
| | Remnant | $3692.98 \pm 48.50_{a}$ |
| Phosphorus metabolism | Degraded | $37.78 \pm 0.53_{a}$ |
| | Regenerated | $39.01 \pm 1.55_{ab}$ |
| | Revegetated | $37.99 \pm 1.00_{a}$ |
| | Remnant | $40.81 \pm 1.47_{b}$ |
| Iron acquisition and metabolism | Degraded | $51.90 \pm 1.77_{a}$ |
| | Regenerated | $52.68 \pm 3.15_{a}$ |
| | Revegetated | $50.33 \pm 2.85_{a}$ |
| | Remnant | $51.69 \pm 3.48_{a}$ |
| Nitrogen metabolism | Degraded | $35.77 \pm 2.68_{a}$ |

| | | |
|---|---|---|
| | Regenerated | $35.93 \pm 2.82_{a}$ |
| | Revegetated | $32.77 \pm 2.08_{a}$ |
| | Remnant | $34.50 \pm 3.61_{a}$ |
| Sulphur acquisition and metabolism | Degraded | $87.88 \pm 2.87_{a}$ |
| | Regenerated | $91.29 \pm 3.73_{a}$ |
| | Revegetated | $87.66 \pm 1.86_{a}$ |
| | Remnant | $90.57 \pm 3.81_{a}$ |
| Amino acids and derivatives | Degraded | $431.86 \pm 7.24_{a}$ |
| | Regenerated | $439.54 \pm 10.46_{a}$ |
| | Revegetated | $430.05 \pm 5.73_{a}$ |
| | Remnant | $438.39 \pm 11.21_{a}$ |
| Carbohydrates | Degraded | $616.92 \pm 13.79_{a}$ |
| | Regenerated | $631.50 \pm 18.62_{a}$ |
| | Revegetated | $618.48 \pm 10.74_{a}$ |
| | Remnant | $633.10 \pm 14.93_{a}$ |
| Clustering-based subsystems | Degraded | $238.25 \pm 6.74_{a}$ |
| | Regenerated | $245.77 \pm 9.88_{a}$ |
| | Revegetated | $241.08 \pm 5.45_{a}$ |
| | Remnant | $243.80 \pm 4.40_{a}$ |
| DNA metabolism | Degraded | $2380.04 \pm 256.24_{ab}$ |
| | Regenerated | $2339.50 \pm 86.20_{ab}$ |
| | Revegetated | $2094.29 \pm 246.05_{a}$ |
| | Remnant | $2312.63 \pm 101.58_{b}$ |

| | | |
|---|---|---|
| Regulation and cell signalling | Degraded | $85.89 \pm 3.16_{a}$ |
| | Regenerated | $87.86 \pm 5.46_{a}$ |
| | Revegetated | $87.93 \pm 3.71_{a}$ |
| | Remnant | $87.74 \pm 6.81_{a}$ |

**Table S3** Permutational multivariate analysis of variance (PERMANOVA) *p* values from tests assessing differences in either community (taxonomy) or functional (all others) compositions (beta diversity) across our four land conditions and six sites. Bolded values indicate significant differences ($p < 0.05$) across either land condition or site.

| Group | Land Condition | Site |
|---|---|---|
| Taxonomy | **0.009** | **0.029** |
| All functions | **0.015** | **0.006** |
| Phosphorus metabolism | 0.079 | **0.001** |
| Iron acquisition and metabolism | 0.07 | **0.002** |
| Nitrogen metabolism | **0.041** | **0.011** |
| Sulphur acquisition and metabolism | **0.021** | **0.022** |
| Amino acids and derivatives | **0.023** | **0.008** |
| Carbohydrates | 0.076 | **0.003** |
| Clustering-based subsystems | **0.011** | **0.049** |
| DNA metabolism | **0.02** | 0.083 |
| Regulation and cell signalling | **0.006** | 0.158 |

**Table S4** Log fold change (LFC) values for differently abundant phosphorus metabolism functions from pairwise comparisons of each land condition all to the remnant condition.

| Function | Comparison | LFC |
|---|---|---|
| Phosphonate utilization as. acetyltransferase | Revegetated | -0.930 |
| Phosphonate utilization as. acetyltransferase | Regenerated | -0.212 |
| Phosphonate utilization as. acetyltransferase | Degraded | -0.779 |
| Ribose 1,5 biphosphate phosphokinase | Revegetated | -0.883 |
| Ribose 1,5 biphosphate phosphokinase | Regenerated | -0.560 |
| Ribose 1,5 biphosphate phosphokinase | Revegetated | -0.876 |

# Appendix 3:

# Supplementary material from Chapter 4 *Stronger together: intact soil translocation increases the resilience of inoculated microbial communities*

Shawn D. Peddle[1*], Christian Cando-Dumancela[1], Tarryn C. Davies[1], Robert Edwards[1], Riley J. Hodgson[1], Siegfried L. Krauss[2,3], Craig Liddicoat[1], Angela Sanders[4], Martin F. Breed[1]

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[3]The University of Western Australia, Crawley, WA, Australia.

[4]Bush Heritage Australia, Albany, WA, Australia.

## 1. Supplementary methods

### *1.1 DNA Extraction, Sequencing and Bioinformatics*

For DNA extractions for both T0 and T1 samples, we used the Qiagen DNeasy PowerLyzer PowerSoil Kit following the manufacturer's instructions and quantified concentrations fluorometrically. DNA extractions for T0 samples were conducted in October 2022 and DNA was sent to the Australian Genome Research Facility (AGRF) in Melbourne, Australia for sequencing. Soil bacterial 16S rRNA from the V3-V4 region was amplified using the 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) primer set (Yu *et al.* 2005), and for fungi, the internal transcribed spacer (ITS) region in the rRNA operon was amplified using the 1F and 2R primers before 300 base pair paired end sequencing on the Illumina MiSeq platform.

DNA extractions for the T1 samples were conducted in June 2023 and DNA was again sent to AGRF for sequencing, this time using the Illumina NexSeq 2000 platform. The same gene regions (16S V3-V4 and ITS 1F-2R) were amplified using the same primer sets as the T0 sequencing. T0 and T1 sequencing generated four FastQ format datasets (T0 16S, T0 ITS, T1 16S, T1 ITS) for bioinformatic processing.

Due to the different sequencing platforms used across the two sample events, bioinformatics were conducted individually on each dataset using the *DADA2* pipeline (Callahan *et al.* 2016) up to the chimera removal step. For the T0 16S and ITS datasets, FASTQ files were quality checked, and primer sequences removed using *Cutadapt* (Martin 2011) and trimming lengths were determined for forward and

reverse reads using *FIGARO* (Weinstein *et al.* 2019). Forward and reverse reads were trimmed, error rates inferred, paired end reads merged, and sequence tables constructed with *DADA2*.

For the T1 16S rRNA and ITS datasets, as the Illumina NextSeq platform generates binned quality scores where both *FIGARO* and *DADA2* expect non-binned quality scores, methods differ as follows: Instead of using *FIGARO* to determine trimming parameters, the forward and reverse read quality profiles were generated and visualised with *DADA2*; and at the error estimation steps, monotonicity was enforced, and loess smoothing was applied to improve error rate modelling before sequence tables were constructed. At this point the T0 and T1 sequence tables were then merged (16S rRNA and ITS data handled separately) before chimera removal and taxonomic assignment. Taxonomy was assigned to the sequence tables using QIIME2 with the Silva database (v138.1) (Wang *et al.* 2007) for 16S rRNA data and UNITE (v9.0) (Abarenkov *et al.* 2023) for ITS data using a naïve Bayesian classifier to produce separate 16S rRNA and ITS amplicon sequence variant (ASV) abundance tables. All further statistical analyses were conducted using R (Version 4.4.0; R core team 2024), using the *Phyloseq* (McMurdie & Holmes 2013) package to combine sample metadata with ASV abundance and taxonomy tables.

*1.2 R Packages used for analysis*

We used the following R packages during our analysis: *Phyloseq* (McMurdie & Holmes 2013), *vegan* (Oksanen *et al.* 2013), *adonis2* (Oksanen *et al.* 2013), *lmPerm* (Wheeler *et al.* 2016), *ancombc2* (Lin & Peddada 2024), *caret* (Kuhn, 2015).

## Supplementary figures

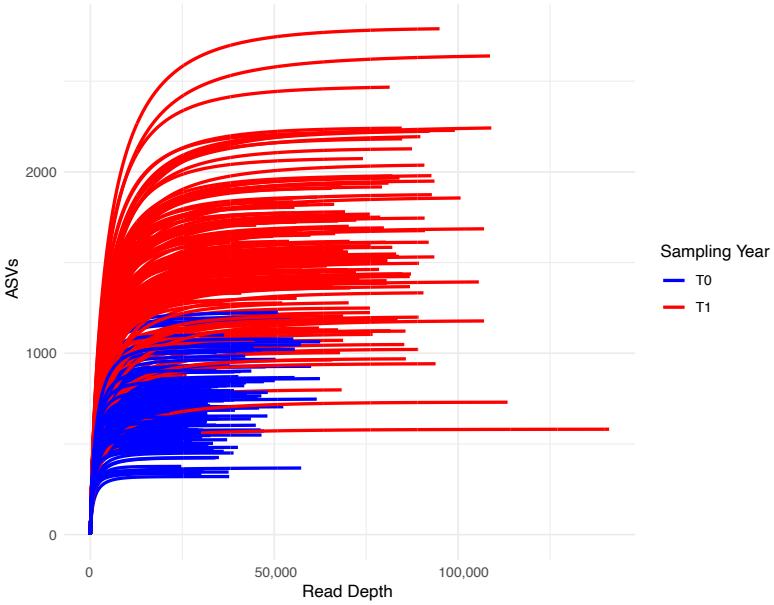


**Figure S1** Rarefaction curve of 16s amplicon sequence read depth by number of ASVs from both the T0 and T1 sampling events used to determine appropriate level of rarefaction and ensure ASV abundance was adequately represented at the chosen level of rarefaction (20,717 reads).

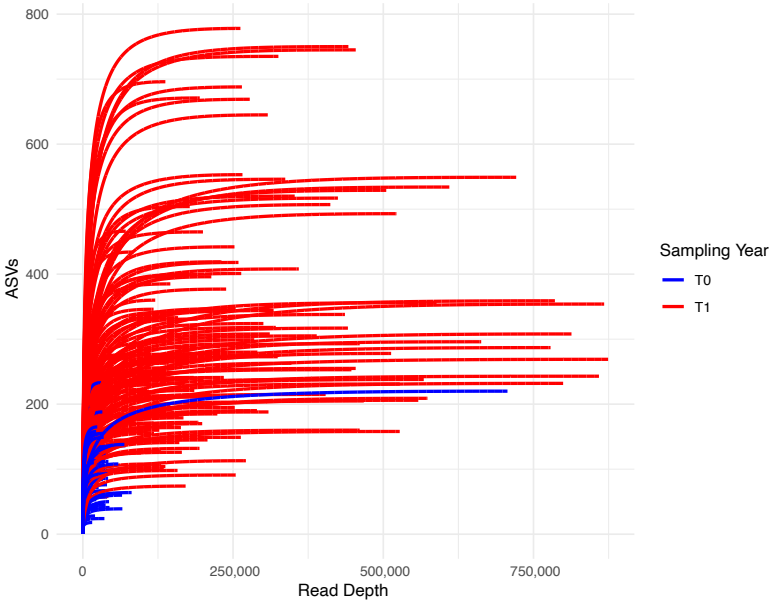

**Figure S2** Rarefaction curve of ITS amplicon sequence read depth by number of ASVs from both the T0 and T1 sampling events used to determine appropriate level of rarefaction and ensure ASV abundance was adequately represented at the chosen level of rarefaction (10,073 reads).

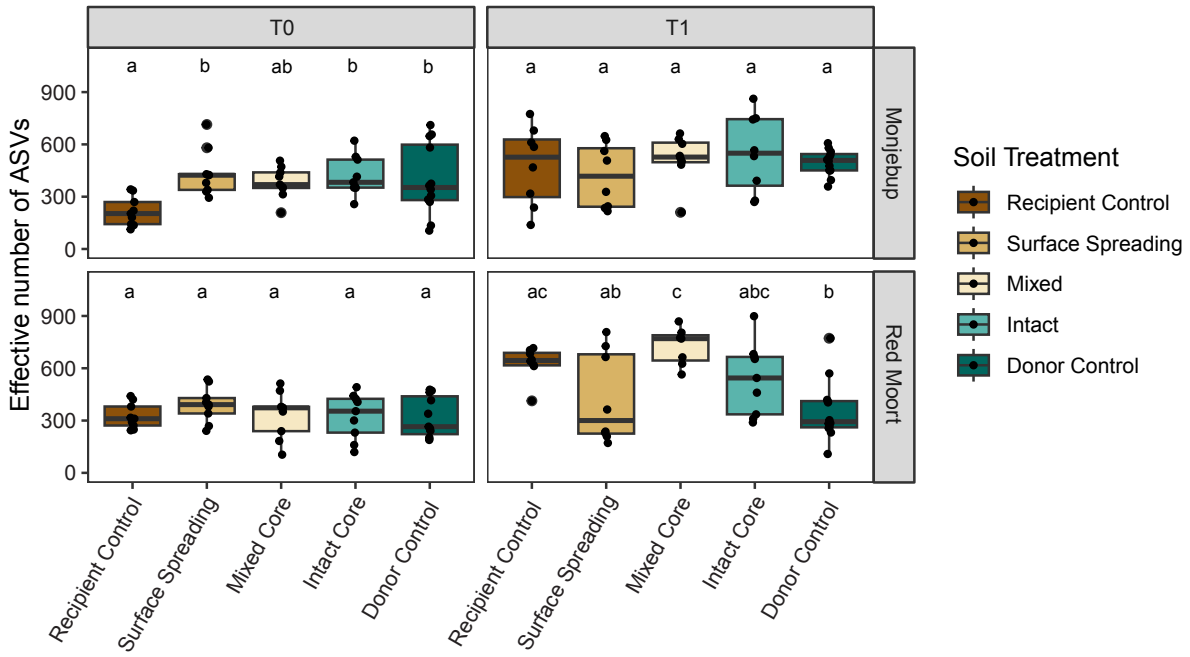


**Figure S3** Boxplots of bacterial alpha diversity (effective number of ASVs) for each soil translocation treatment across each sample year (T0, T1) and site (Monjebup, Red Moort). Groups that do not share a letter significantly differ ($p < 0.05$, ANOVA).

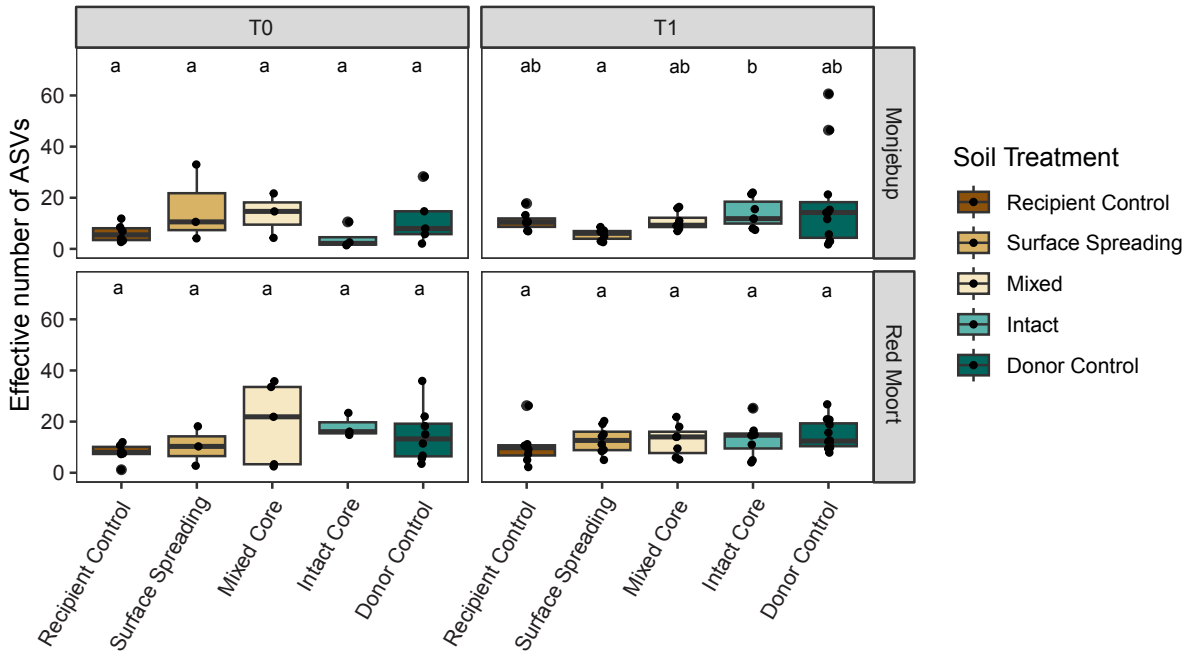


**Figure S4** Boxplots of fungal alpha diversity (effective number of ASVs) for each soil translocation treatment across each sample year (T0, T1) and site (Monjebup, Red Moort). Groups that do not share a letter significantly differ ($p < 0.05$, ANOVA).

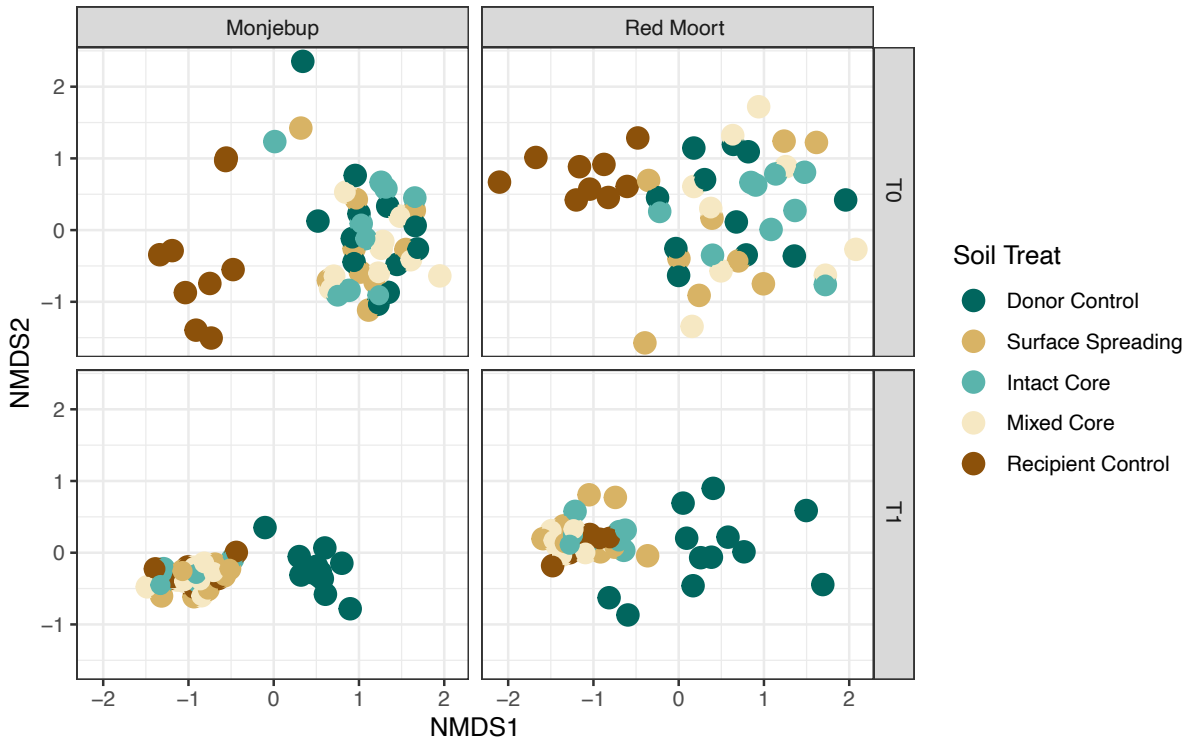

**Figure S5.** Non-metric multidimensional scaling (NMDS) ordination of bacterial (16s) community composition faceted by Site and Sample Year from original sampling at T0 and dispersal samples collected at T1 to assess dispersal of translocated bacteria into the surrounding soil.

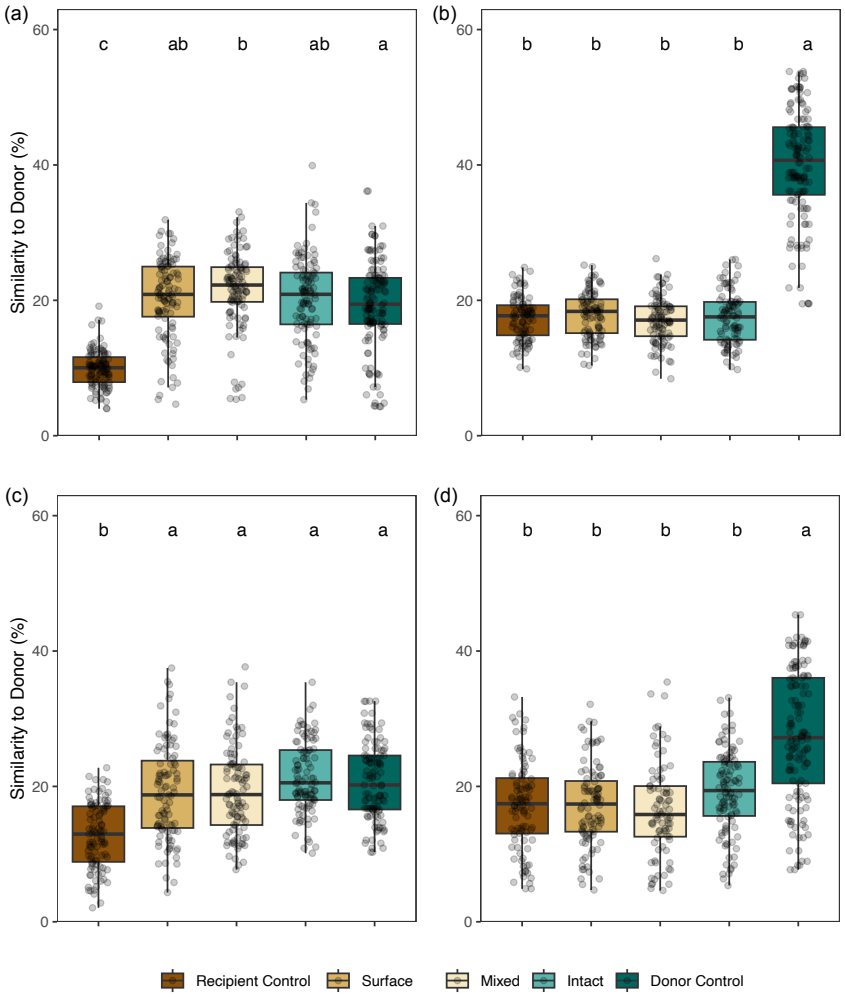


**Figure S6** Similarity to donor boxplots for 16s dispersal samples. (a) Similarity of bacterial communities at Monjebup across the three soil treatments and two controls to donor control communities at the time of soil translocation (T0). (b) Similarity of bacterial communities at Monjebup from soil samples collected from surrounding un-translocated soil one year pot-translocation (T1) to assess dispersal of soil bacteria. (c) Similarity of bacterial communities at Red Moort across the three soil treatments

and two controls to donor control communities at the time of soil translocation (T0). (d) Similarity of bacterial communities at Red Moort from soil samples collected from surrounding un-translocated soil one year post-translocation (T1) to look for dispersal of soil bacteria.

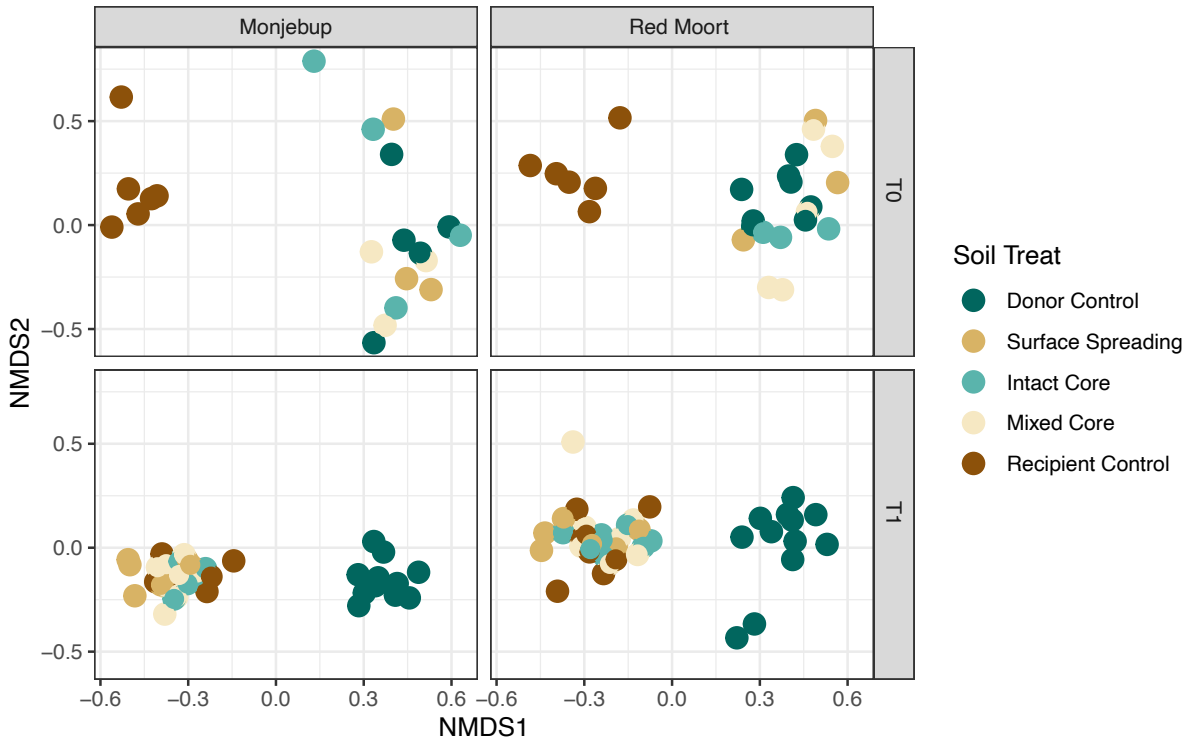


**Figure S7** Non-metric multidimensional scaling (NMDS) ordination of fungal (ITS) community composition faceted by Site and Sample Year from original sampling at T0 and dispersal samples collected at T1 to assess dispersal of translocated fungi into the surrounding soil.

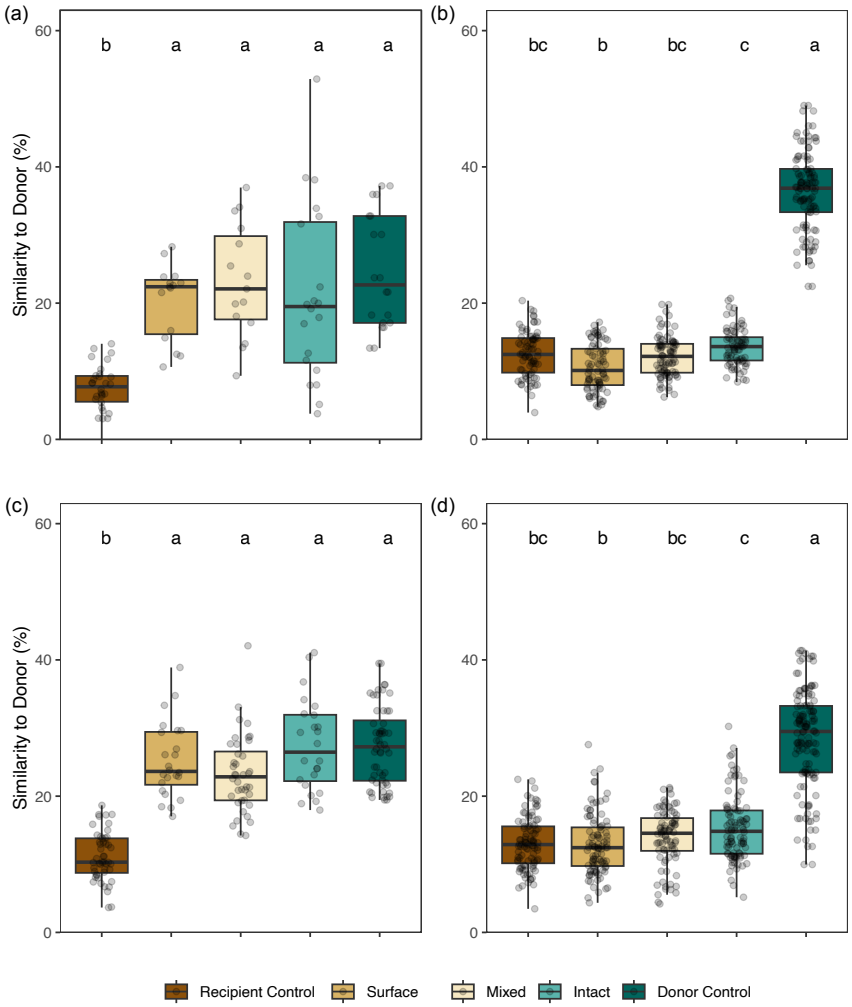


**Figure S8** Similarity to donor boxplots for ITS dispersal samples. (a) Similarity of fungal communities at Monjebup across the three soil treatments and two controls to donor control communities at the time of soil translocation (T0). (b) Similarity of fungal communities at Monjebup from soil samples collected from surrounding un-translocated soil to look for dispersal of soil bacteria. (c) Similarity of fungal communities at Red Moort across the three soil treatments and two controls to donor control communities at the time of soil translocation (T0). (d) Similarity of fungal communities at Red Moort from soil samples collected from surrounding un-translocated soil to look for dispersal of soil fungi.

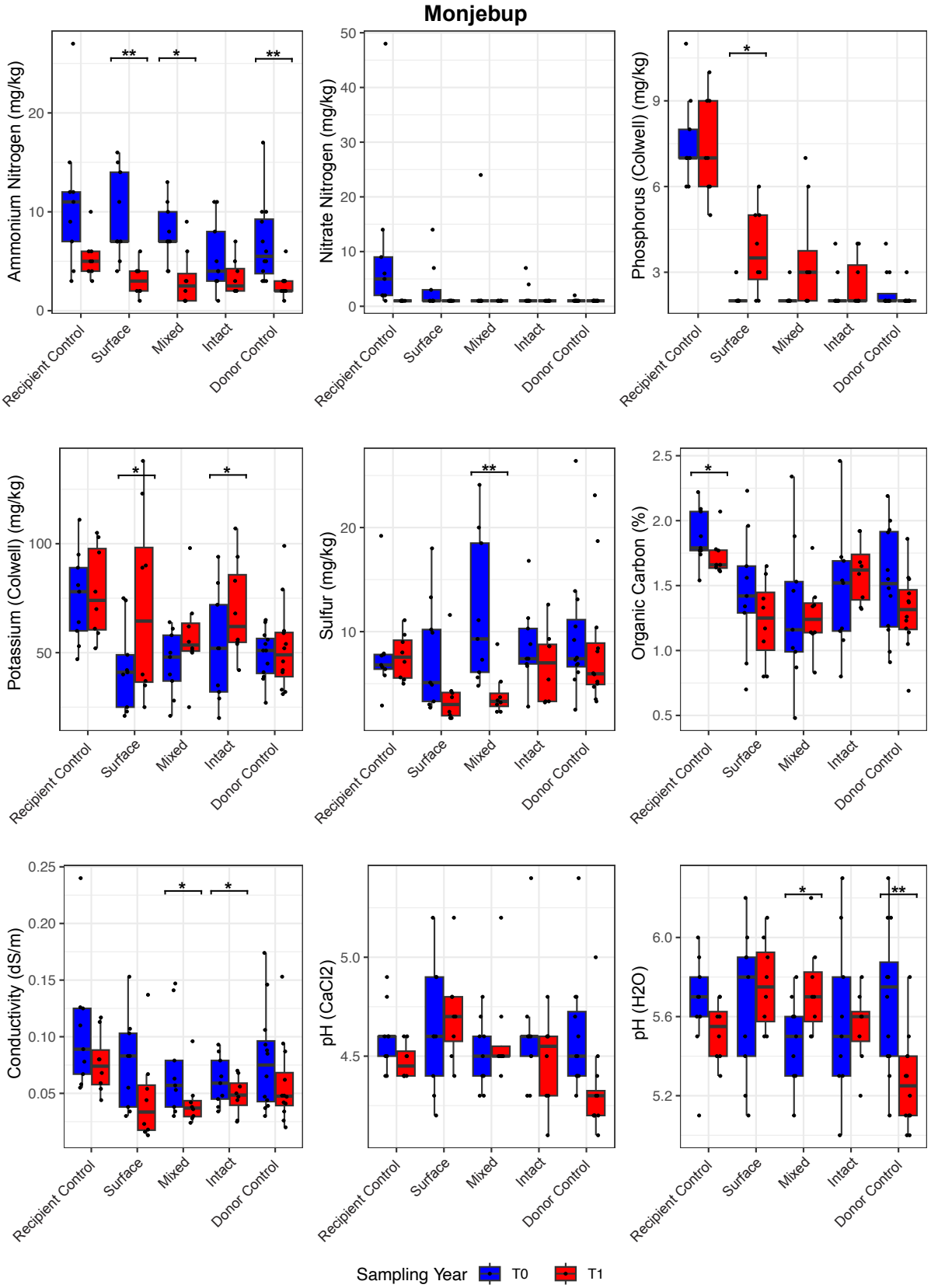
Monjebup
Ammonium Nitrogen (mg/kg)
Nitrate Nitrogen (mg/kg)
Phosphorus (Colwell) (mg/kg)
Potassium (Colwell) (mg/kg)
Sulfur (mg/kg)
Organic Carbon (%)
Conductivity (dS/m)
pH (CaCl2)
pH (H2O)
Recipient Control
Surface
Mixed
Intact
Donor Control
Sampling Year
T0
T1

**Figure S9** Boxplots of measured soil abiotic properties at Monjebup across soil translocation treatments and the two sample years. Asterixis indicate significant differences ($p < 0.05$, paired t-test) in abiotic properties between sample years.

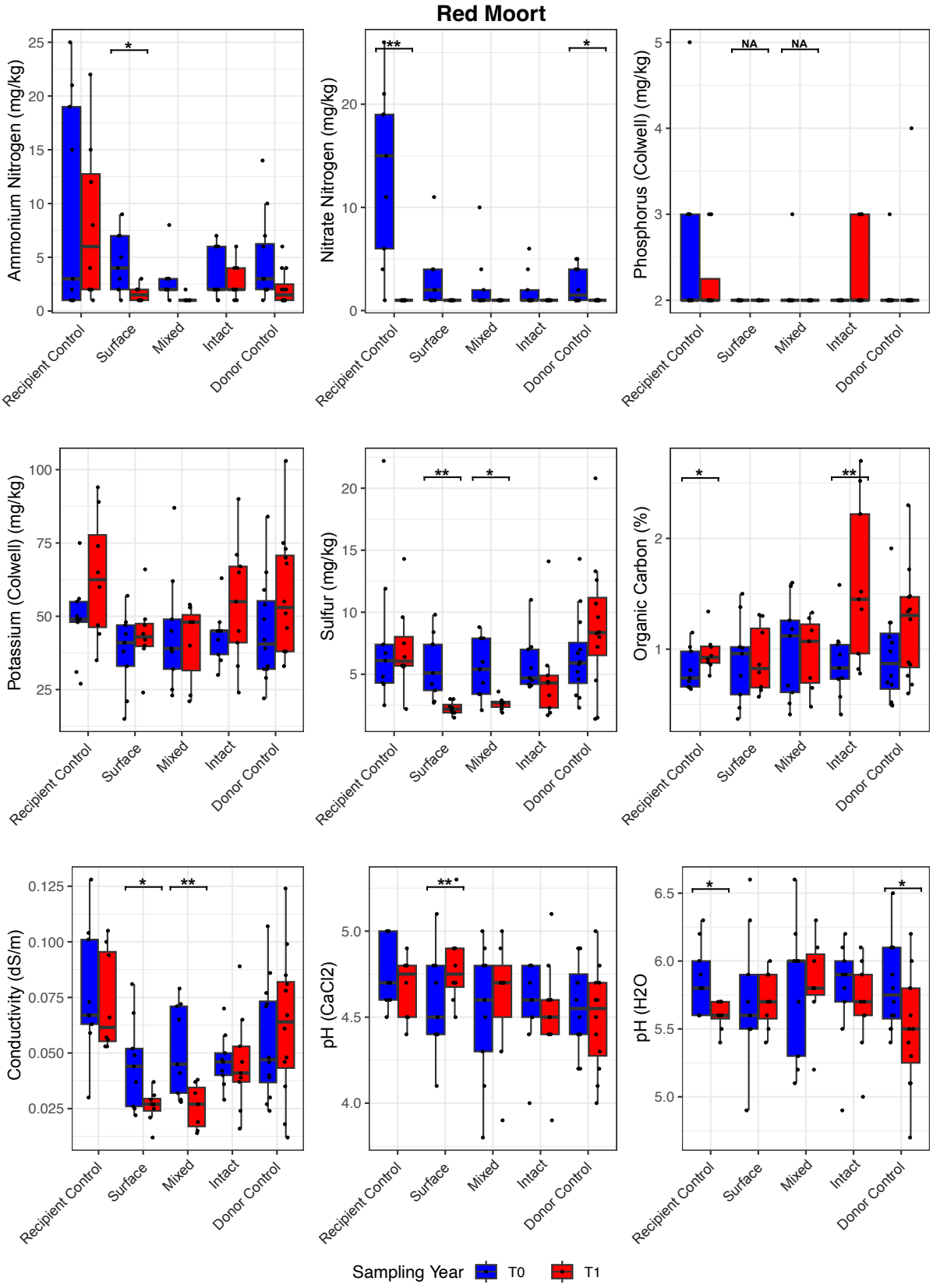
Red Moort
Ammonium Nitrogen (mg/kg)
Nitrate Nitrogen (mg/kg)
Phosphorus (Colwell) (mg/kg)
Potassium (Colwell) (mg/kg)
Sulfur (mg/kg)
Organic Carbon (%)
Conductivity (dS/m)
pH (CaCl2)
pH (H2O)
Recipient Control
Surface
Mixed
Intact
Donor Control
NA
Sampling Year
T0
T1

**Figure S10** Boxplots of measured soil abiotic properties at Red Moort across soil translocation treatments and the two sample years. Asterixis indicate significant differences ($p < 0.05$, paired t-test) in abiotic properties between sample years.

## 3 Supplementary tables

**Table S1** Model coefficients from PERMANOVAs of bacterial community compositions stratified within each combination of Site and Sample Year.

| Factor | *Df* | *SS* | $R^2$ | *F* | *P* |
|---|---|---|---|---|---|
| Soil Treatment | 4 | 6.021 | 0.099 | 5.33 | 0.001 |
| Site | 1 | 2.278 | 0.037 | 8.067 | 0.001 |
| Sample Year | 1 | 2.555 | 0.0421 | 9.074 | 0.001 |
| Residual | 176 | 49.704 | 0.820 | | |
| Total | 182 | 60.558 | 1.000 | | |

**Table S2** Model coefficients from PERMANOVAs of fungal community compositions stratified within each combination of Site and Sample Year.

| Factor | *Df* | *SS* | $R^2$ | *F* | *P* |
|---|---|---|---|---|---|
| Soil Treatment | 4 | 5.941 | 0.108 | 3.954 | 0.001 |
| Site | 1 | 1.665 | 0.030 | 4.433 | 0.001 |
| Sample Year | 1 | 1.156 | 0.0210 | 3.077 | 0.001 |
| Residual | 123 | 46.192 | 0.840 | | |
| Total | 129 | 54.954 | 1.000 | | |

**Table S3** Mean (± SD) similarity to donor values for bacterial (16S rRNA) communities at both Monjebup and Red Moort for both sampling years. Similarity to donor values within each site/sample year combination that do not share a letter significantly differ (Dunn, $p < 0.05$).

| Site | Sample Year | Soil Treatment | Similarity to Donor (%) (mean ± SD) |
|---|---|---|---|
| Monjebup | T0 | Recipient Control | $9.89 \pm 2.71_{b}$ |
| | | Surface Spreading | $20.3 \pm 5.94_{a}$ |
| | | Mixed Core | $21.6 \pm 5.69_{a}$ |
| | | Intact Core | $20.2 \pm 6.25_{a}$ |
| | | Donor Control | $19.3 \pm 6.64_{a}$ |
| | T1 | Recipient Control | $17.2 \pm 3.54_{d}$ |
| | | Surface Spreading | $29.6 \pm 8.34_{b}$ |
| | | Mixed Core | $33.1 \pm 7.30_{bc}$ |
| | | Intact Core | $37.1 \pm 7.12_{ac}$ |
| | | Donor Control | $39.9 \pm 8.34_{a}$ |
| Red Moort | T0 | Recipient Control | $12.7 \pm 4.88_{b}$ |
| | | Surface Spreading | $19.1 \pm 7.13_{a}$ |
| | | Mixed Core | $19.3 \pm 6.55_{a}$ |
| | | Intact Core | $21.3 \pm 5.26_{a}$ |
| | | Donor Control | $20.7 \pm 5.72_{a}$ |
| | T1 | Recipient Control | $19.3 \pm 7.51_{b}$ |
| | | Surface Spreading | $19.9 \pm 8.54_{b}$ |
| | | Mixed Core | $25.6 \pm 8.48_{a}$ |
| | | Intact Core | $27.6 \pm 8.56_{a}$ |
| | | Donor Control | $27.5 \pm 9.81_{a}$ |

**Table S4** Mean (± SD) similarity to donor values for fungal (ITS) communities at both Monjebup and Red Moort for both sampling years. Similarity to donor values within each site/sample year combination that do not share a letter significantly differ (Dunn, $p < 0.05$).

| Site | Sample Year | Soil Treatment | Similarity to Donor (%) (mean ± SD) |
|---|---|---|---|
| Monjebup | T0 | Recipient Control | $7.55 \pm 3.36_{b}$ |
| | | Surface Spreading | $20.3 \pm 5.57_{a}$ |
| | | Mixed Core | $23.2 \pm 8.31_{a}$ |
| | | Intact Core | $21.2 \pm 13.0_{a}$ |
| | | Donor Control | $24.7 \pm 8.71_{a}$ |
| | T1 | Recipient Control | $12.2 \pm 3.90_{d}$ |
| | | Surface Spreading | $27.0 \pm 5.31_{b}$ |
| | | Mixed Core | $30.3 \pm 5.77_{bc}$ |
| | | Intact Core | $32.2 \pm 8.15_{c}$ |
| | | Donor Control | $36.9 \pm 5.85_{a}$ |
| Red Moort | T0 | Recipient Control | $11.3 \pm 3.56_{b}$ |
| | | Surface Spreading | $25.1 \pm 5.62_{a}$ |
| | | Mixed Core | $23.6 \pm 5.71_{a}$ |
| | | Intact Core | $27.6 \pm 6.93_{a}$ |
| | | Donor Control | $27.4 \pm 5.48_{a}$ |
| | T1 | Recipient Control | $15.7 \pm 7.36_{d}$ |
| | | Surface Spreading | $19.3 \pm 5.29_{b}$ |
| | | Mixed Core | $25.5 \pm 6.37_{ac}$ |
| | | Intact Core | $24.1 \pm 8.83_{c}$ |
| | | Donor Control | $28.3 \pm 7.78_{a}$ |

**Table S5** Mean (± SD) effective number of bacterial (16S) amplicon sequence variants (ASV) for each soil translocation treatment across both sample years (T0, T1) and sites (Monjebup, Red Moort). Soil treatments not sharing a letter within each site/sample year combination have significantly different effective numbers of ASVs (Monjebup T0: Tukey, $p < 0.05$; Monjebup T1: Dunn, $p < 0.05$; Red Moort T0: Tukey, $p < 0.05$; Red Moort T1: Tukey, $p < 0.05$).

| Site | Sample Year | Soil Treatment | Effective no. ASVs |
|---|---|---|---|
| Monjebup | T0 | Recipient Control | $215.79 \pm 84.16_{a}$ |
| | | Surface Spread | $434.85 \pm 133.61_{b}$ |
| | | Mixed | $381.1 \pm 90_{ab}$ |
| | | Intact | $419.76 \pm 113.02_{b}$ |
| | | Donor Control | $398.13 \pm 204.25_{b}$ |
| | T1 | Recipient Control | $476.53 \pm 225.82_{a}$ |
| | | Inoculation | $420.89 \pm 183.83_{a}$ |
| | | Mixed | $518.34 \pm 139.96_{a}$ |
| | | Intact | $549.04 \pm 224.57_{a}$ |
| | | Donor Control | $496.88 \pm 74.03_{a}$ |
| Red Moort | T0 | Recipient Control | $324.85 \pm 72.75_{a}$ |
| | | Inoculation | $390.63 \pm 100.23_{a}$ |
| | | Mixed | $331.89 \pm 132.61_{a}$ |
| | | Intact | $325.4 \pm 131.24_{a}$ |
| | | Donor Control | $319.35 \pm 117.29_{a}$ |
| | T1 | Recipient Control | $629.43 \pm 95.38_{ac}$ |
| | | Inoculation | $426.27 \pm 262.62_{ab}$ |
| | | Mixed | $724.36 \pm 108.67_{c}$ |
| | | Intact | $537.57 \pm 206.42_{abc}$ |

| | | |
|---|---|---|
| | Donor Control | 358.57 ± 174.99$_b$ |

**Table S6** Mean (± SD) effective number of fungal (ITS) amplicon sequence variants (ASV) for each soil translocation treatment across both sample years (T0, T1) and sites (Monjebup, Red Moort). Soil treatments not sharing a letter within each site/sample year combination have significantly different effective numbers of ASVs (Monjebup T0: Tukey, $p < 0.05$; Monjebup T1: Dunn, $p < 0.05$; Red Moort T0: Tukey, $p < 0.05$; Red Moort T1: Tukey, $p < 0.05$).

| Site | Samp Year | Soil Treat | Effective no. ASVs |
|---|---|---|---|
| Monjebup | T0 | Recipient Control | $6.20 \pm 3.55_{a}$ |
| | | Inoculation | $15.89 \pm 15.16_{a}$ |
| | | Mixed | $13.55 \pm 8.75_{a}$ |
| | | Intact | $4.07 \pm 4.35_{a}$ |
| | | Donor Control | $11.76 \pm 10.32_{a}$ |
| | T1 | Recipient Control | $10.87 \pm 3.73_{ab}$ |
| | | Inoculation | $5.58 \pm 2.19_{a}$ |
| | | Mixed | $10.66 \pm 3.59_{ab}$ |
| | | Intact | $14 \pm 5.91_{b}$ |
| | | Donor Control | $17.92 \pm 18.92_{ab}$ |
| Red Moort | T0 | Recipient Control | $7.84 \pm 3.75_{a}$ |
| | | Inoculation | $10.4 \pm 7.71_{a}$ |
| | | Mixed | $19.4 \pm 15.97_{a}$ |
| | | Intact | $18.09 \pm 4.64_{a}$ |
| | | Donor Control | $14.8 \pm 10.68_{a}$ |
| | T1 | Recipient Control | $10.26 \pm 7.156_{a}$ |
| | | Inoculation | $12.75 \pm 5.33_{a}$ |
| | | Mixed | $12.64 \pm 6.16_{a}$ |

| | | |
|---|---|---|
| | Intact | 13.23 ± 6.76$_a$ |
| | Donor Control | 14.77 ± 5.83$_a$ |