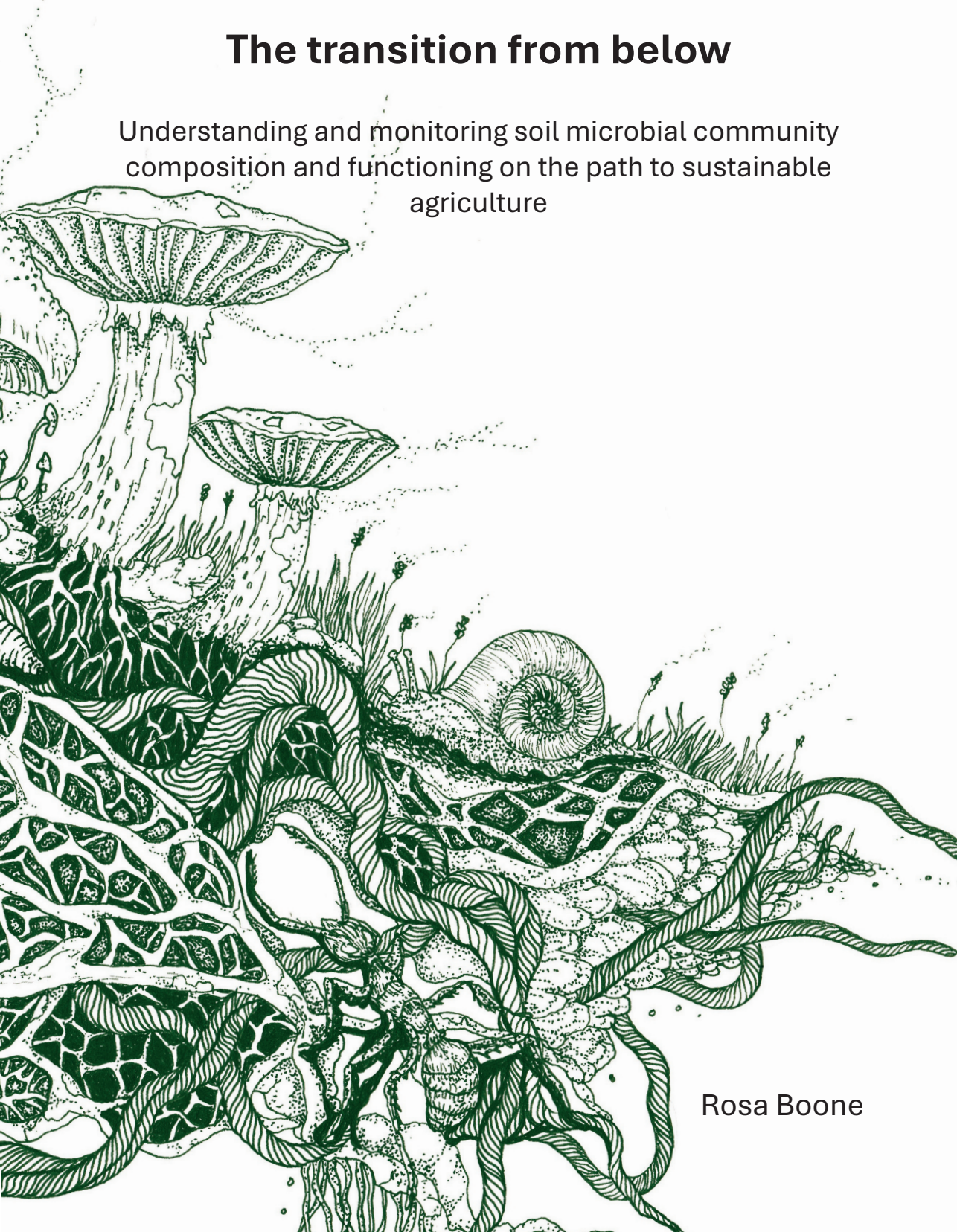
The transition from below

Understanding and monitoring soil microbial community composition and functioning on the path to sustainable agriculture



Rosa Boone

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R.W.C Boone

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geboren op 26 september 1995
te Zutphen

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DISSERTATION

to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. J.M. Sanders,
according to the decision of the Doctorate Board
to be defended in public on
on Tuesday, September 29, 2026
at 2:30 pm

by

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born on September 26, 1995
in Zutphen, the Netherlands

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Aan de oude en nieuwe Boontjes

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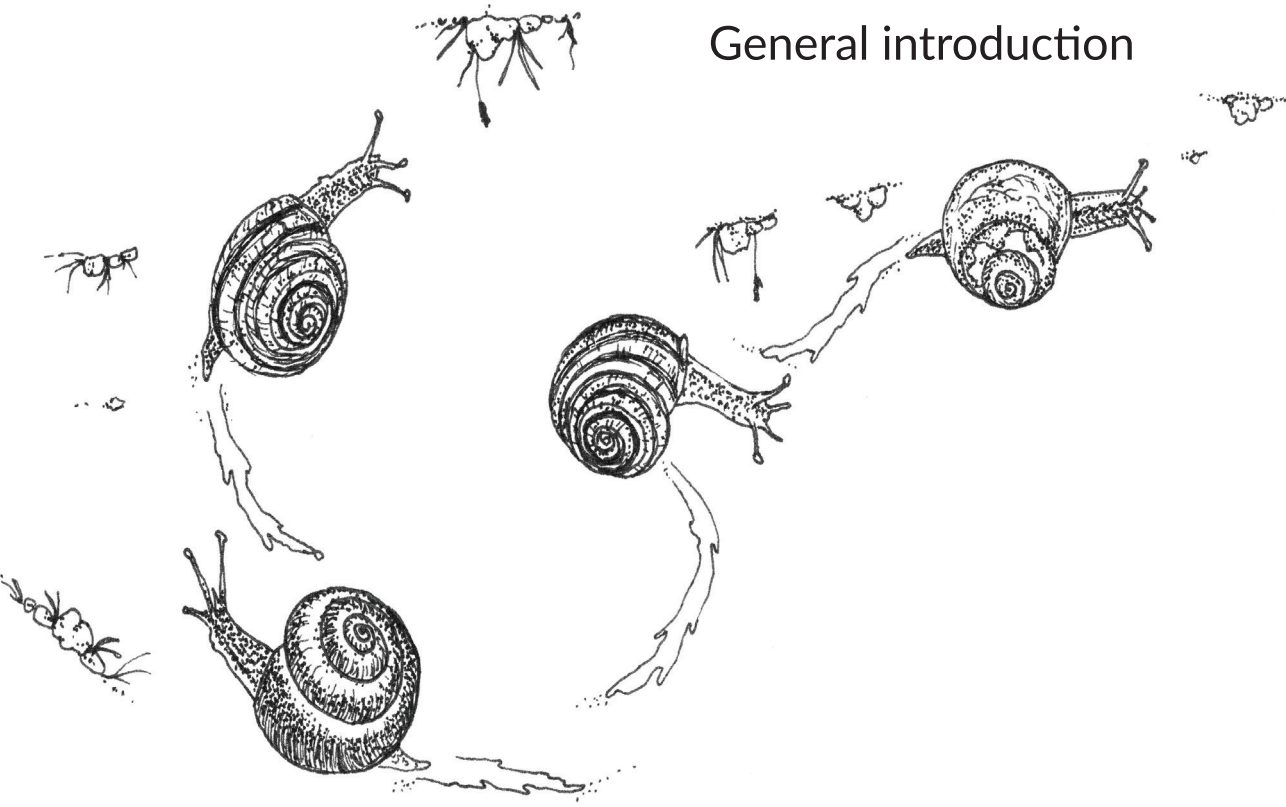
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”When in doubt, always follow your nose”

1

General introduction



1

Farmer: "I think we've started to take more of an interest in, this sounds daft because we should have been doing it for the last 30 years, but we've been taken much more interest in soil [...] [whereas before] we've been concentrating on speed of work and size of machinery and getting it across the land quickly and so on, we now, we've realised that we've hit this sort of yield plateau so our yields have been sort of semi plateauing although not coming down. [...] We just started to look at some of the issues we were having and found that actually most of the fixes were involved in improving the soil."

— From: Caring for soil life in the Anthropocene: The role of attentiveness in more-than-human ethics. (2019). A. Krywoszynska

Agricultural intensification and its limits

The success story of modern agriculture - its capacity to feed billions of people - has recently become the source of its greatest challenge: producing food sustainably without harming the environment. During the second half of the twentieth century, global agricultural intensification, driven by the need to feed a rapidly growing population, has changed ecosystems in an unprecedented manner (Foley et al., 2005). This period of intensification has been enabled by the widespread use of mineral fertilisers, synthetic pesticides, and high-yielding crop varieties developed during the Green Revolution (Conway & Barbier, 1988). While these practices have substantially increased agricultural productivity, they have also led to disproportional use of fertilizers, leading to excess levels of nutrients in surface and ground water and in natural areas (Erisman et al., 2013; Kopittke et al., 2019; Pretty, 2018; Tsiafouli et al., 2015). As a result, the dominant 'high input-high output' production model is now recognised as a major driver of environmental degradation and a key contributor to the transgression of some planetary boundaries, such as the phosphorus and nitrogen cycle (Campbell et al., 2017). The central challenge facing modern agriculture is therefore no longer whether it can produce enough food, but whether food production can be sustained without undermining the ecological systems on which it ultimately depends (Bommarco et al., 2013; Kleijn et al., 2019; Van Grinsven et al., 2015).

The Netherlands, in many ways, exemplifies this global pattern of agricultural intensification and its consequences. Initially promoting food security by enhancing agricultural production, from the mid-twentieth century onward agricultural intensification in the Netherlands became increasingly driven by economic growth and export competitive-

ness (Zwarts et al., 2021) This shift was facilitated by the introduction of artificial fertilizer, the import of animal feed, post-war modernisation policies (including the Common Agricultural Policy (CAP)), and large-scale land consolidation in the 1970's. Today, approximately 54% of the Dutch land surface is devoted to agriculture, with 65% of this land being used for livestock feed production; primarily dairy farming (Centraal Bureau voor de Statistiek (CBS), 2025). This highly specialised and intensive agricultural system generates economic value, contributing approximately 11 billion euros in 2020 of gross added value (around 1.3% of national GDP), and has positioned the Netherlands as a major agricultural exporter within Europe.

The economic success of this Dutch intensive agricultural model has, like many other industrialised countries, been accompanied by profound societal and environmental consequences. The intensive Dutch agricultural system has resulted in nitrogen surpluses and ammonia emissions that are among the highest in Europe (Van Grinsven et al., 2015). The external societal costs associated with agricultural nitrogen losses have been estimated at 0.3-1.9% of the European Gross Domestic Product (GDP) (Van Grinsven et al., 2013). Furthermore, agriculture intensification has dramatically simplified the landscape in the Netherlands. In dairy regions, hedgerows, tree lines, ditches, small parcels, and herb-rich field margins have been systematically removed to facilitate large, uniform agricultural grasslands (Baumgarten et al., 2025; Robinson et al., 2025). These simplified grassland systems, dominated mainly by one single grass species - Lolium perenne L., have led to a substantial loss in above- and belowground biodiversity (Emmerson et al., 2016; Kleijn et al., 2012; Tsiafouli et al., 2015). This loss in above- and belowground biodiversity has been shown to impair the long-term stability and resilience of ecosystems, leading to a degradation of key ecosystem services (Hooper et al., 2012). A commissioned societal cost-benefit analysis estimated the societal costs of the current Dutch agricultural system to outweigh its economic benefits by approximately €5.3 billion per year (Scheele et al., 2025). This has prompted increasing interest in agricultural systems that reduce environmental impacts while maintaining productivity.

Extensification as a systems transition

In highly industrialised agricultural regions, particularly in north-western Europe, the question is how to adjust the current production model so that environmental impacts are reduced while agricultural production remains efficient (MacLaren et al., 2022; Staniszewski et al., 2023). One promising alternative for the current model is agricultural extensification, which aims to reduce environmental impacts and resource depletion, while maintaining, or minimizing, losses in food production (sensu Van Grinsven et al.,

2015). Field practices associated with extensification include reduced fertiliser and manure inputs, lower livestock densities, and decreased nutrient losses to the environment, and are reflected in farming systems such as organic, nature-inclusive, or regenerative agriculture (Giller et al., 2021). If these extensified practices on a field-level are combined with extensifying changes at the system level (such as reductions in overall livestock density and nutrient-use efficiency), extensification has the potential to reduce nitrogen pollution and associated societal costs (Van Grinsven et al., 2015). Extensification should therefore be seen not only as a change in field management practices, but as part of a broader reconfiguration of livestock production systems. In that light, this thesis focuses on the changes that are driven by extensification at the field-level.

Consequences for biodiversity

On a field-level, permanent dairy grasslands are often considered suitable systems for extensification because of their biophysical and management-related characteristics. Unlike annually tilled systems, these grasslands maintain a continuous plant cover and experience limited physical soil disturbance (Glover et al., 2010). By reseeding, their plant community composition can also be maintained, even diversified, relatively easily. In Europe, plant communities from semi-natural grasslands are among the most diverse ecosystems, and they are important habitats for many species (above and belowground). However, intensively managed agricultural grasslands are typically species-poor, often dominated by a small number of highly productive grass species. Hence, there is a high potential for biodiversity improvements on these intensively managed agricultural grasslands (Habel et al., 2013; Smukler et al., 2010; Tsiafouli et al., 2015; Vickery et al., 2001; Wallis de Vries & van Swaay, 2009). Apart from hosting biodiversity, grasslands have the potential to contribute to the delivery of multiple ecosystem services, defined here as the benefits ecosystems provide to human society, including food production, climate regulation, water quality regulation and soil fertility (Millennial Ecosystem Assesment, 2005). Grasslands with richer and functionally diverse biota are often associated with enhanced ecosystem services (Delgado-Baquerizo et al., 2016; Delgado-Baquerizo et al., 2017; Wagg et al., 2014). However, whether such benefits are realised in practice under extensification depends not only on aboveground management changes, but also on ecological constraints shaped by past management. Extensification does not occur on a "blank slate" (Cassman et al., 2016; Ceulemans et al., 2014; de Vries et al., 2012; Meyer et al., 2013). Agricultural soils have inherited legacies of decades of intensification that may limit the capacity of grasslands to deliver ecological benefits (Grange et al., 2022; Kardol et al., 2008). Understanding these constraints requires turning attention belowground.

Importance of the soil Agricultural systems are shaped by decades of management, and their responses to changes in management intensity are often constrained by legacy effects, whereby past land use continues to influence ecosystem properties long after practices have changed (Cramer et al., 2008; Suding et al., 2004; Turley et al., 2020). A key reason for these legacy effects is that soils integrate the cumulative effects of long-term management, retaining persistent signatures in nutrient status, organic matter pools and soil communities (Kulmatiski et al., 2006; McLauchlan, 2006; Postma-Blaauw et al., 2012). Consequently, soils are not passive substrates but active determinants of ecosystem trajectories, as these systems play a central role in regulating plant performance, succession and restoration outcomes (De Vries et al., 2013; van der Putten et al., 2013). Soil communities, in particular, play a central role in this process, as they regulate key soil functions that underpin productivity in low-input systems, including nutrient cycling, organic matter decomposition and soil structure formation (Cramer et al., 2008; Suding et al., 2004; Turley et al., 2020). Yet, despite their potential role in the extensification of agricultural systems, the mechanisms through which management practices shape soil communities and thereby constrain or enable ecosystem functioning remain poorly understood (Bardgett & van der Putten, 2014; Nazaries et al., 2021). Therefore, understanding how on-farm management-related conditions influences key functions, will be essential for designing extensification that reduce the reliance on external inputs, without compromising agricultural productivity. Conceptually, extensification can be understood as a transition between two contrasting modes of agricultural functioning. In intensive systems, productivity is primarily maintained through external inputs such as mineral fertilisers, imported feed and chemical control, while biological regulation plays a relatively minor role. In contrast, extensive systems rely more strongly on internally regulated ecological processes, including nutrient cycling mediated by soil organisms, biological pest control and soil structure formation (Bommarco et al., 2013, 2013; Kleijn et al., 2019; Tittonell, 2014). Transitioning from the former to the latter therefore requires not only changes in aboveground management, but also fundamental changes in belowground functioning (Bender et al., 2016). In this sense, extensification represents a long-term soil transition: a shift from externally driven productivity toward systems that rely increasingly on internally regulated soil processes. This thesis approaches extensification through three interlinked questions. This thesis approaches extensification through three interlinked questions (Fig. 1.1): 1) what does long-term extensive management look like belowground; 2) how can this transition be detected and monitored; and 3) can it be actively accelerated? At the core lies the foundational question: what do soil properties, microbial communities, and soil functioning look like across a gradient from conventional to semi-natural management? Chapters 2 and 3 establish

1

what an extensively managed soil system actually looks like belowground. Building on this, Chapters 4 and 5 ask how these changes can be captured and monitored through soil processes and novel indicators. Finally, Chapter 6 asks whether the transition toward extensification can be steered, for instance through soil inoculation, rather than to emerge on its own. Together, these questions frame extensification not merely as a shift in land use, but as a measurable and potentially steerable ecological transition in soil systems.

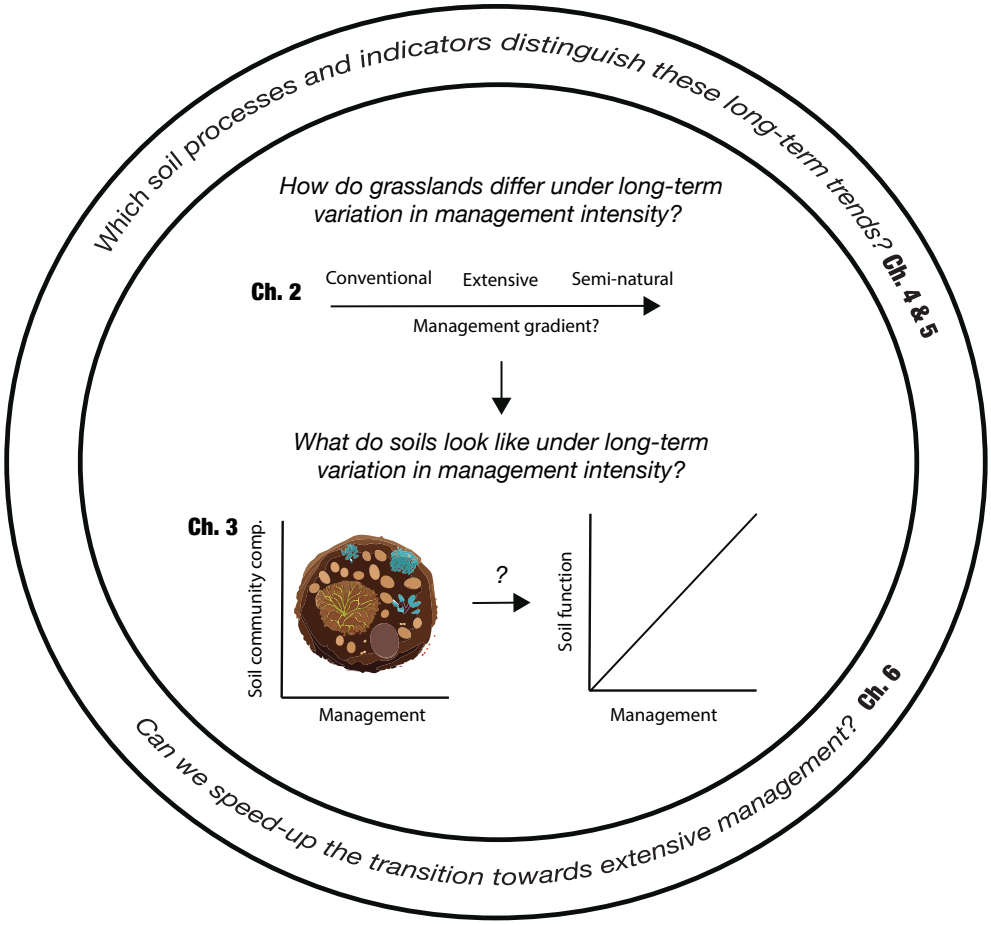


Figure 1.1: Conceptual framework of this thesis. Extensification is framed as a soil transition along a management gradient. Changes in management intensity alter physical, chemical and biological soil properties, which jointly regulate soil functioning and ecosystem services. The thesis investigates (1) how soil state and functioning change under long-term management (core), (2) how these changes can be monitored using process-based metrics and microbial proxies (mVOCs), and (3) whether soil inoculation can accelerate recovery toward a more extensive soil state (outer circle).

Soil properties, communities and their functioning

The 'legacy' of past intensification in grassland soils is stored in the physical, chemical and biological soil properties (van der Putten et al., 2013). These properties differ strongly in their responsiveness to management change and jointly determine how soils function under extensification. Some properties reflect long-term management legacies, while others respond more dynamically to current management. Understanding which soil properties and processes change under extensification, and how these changes translate into altered ecosystem functioning, is therefore essential for evaluating the ecological consequences of management change on field-level. From a physical perspective, soil texture and structure are two key properties that differ markedly in their responsiveness to changes in management. Texture refers to the size and distribution of mineral soil particles, which largely determines the potential for nutrient and organic matter retention (Delgado & Gómez, 2024; Hassink, 1997; Six et al., 2002). As an inherent soil property, texture cannot be modified by management practices. In contrast, soil structure describes the spatial arrangement of soil particles, pores and organic matter within the soil matrix (Delgado & Gómez, 2024). Soil structure in grasslands responds strongly to management through grazing and machinery. High grazing intensity increases mechanical loading through trampling, which compacts soils, increases bulk density, and reduces pore space for air and water movement, leading to poorer soil structural condition and reduced drainage (Hargreaves et al., 2019) (Hargreaves et al., 2019). In contrast, management practices that enhance organic matter inputs, such as manure return through grazing or organic fertilisation, are expected to improve soil structure by promoting aggregate formation and stabilisation through biological activity (e.g. Abdollahi et al., 2014).

From a chemical perspective, properties such as soil organic matter (SOM) content and soil acidity are strongly shaped by changes in management practices. SOM, in particular, is often considered a key component of soils because it both reflects chemical fertility (nutrient availability) and contributes to physical aggregation and biological activity (Biswas & Kole, 2018). Changes in fertilisation regimes, manure inputs and biomass removal directly affect SOM stocks by altering the balance between organic matter inputs and decomposition. A further key chemical property is soil acidity which can be directly altered through liming and indirectly via different inputs of organic matter. Soil acidity regulates nutrient availability for plants and soil organisms (Kemmitt et al., 2005; Song et al., 2023). Together with the physical properties, chemical properties such as SOM and soil acidity define the environment in which soil organisms operate by controlling resource availability, energetic constraints and habitat suitability. As a result, these properties mediate how soil communities respond to changes in management intensity and play a central role in

determining how soils respond to extensification.

The biological properties of the soil include its soil communities, which are known for their diversity and complexity (Decaëns, 2010; Köberl et al., 2020). Over recent decades, ecologists have begun to open up the belowground 'black-box' (Cortois & de Deyn, 2012; Kardol et al., 2015; Mishra et al., 2023; Tiedje et al., 1999). Within the soil system, billions of individual organisms are present. Their size ranges from larger organisms such as earthworms and ants, to arthropods, to the smallest microscopic nematodes, and bacteria and fungi (Bardgett & van der Putten, 2014). Agricultural practices are known to alter multiple groups within the soil community. A Europe-wide survey showed that intensively managed fields exhibit lower soil food web complexity, reduced fungal abundance and higher proportions of plant pathogens (Tsiafouli et al., 2015). Fertilisation regimes, grazing pressure, and biomass removal alter organic matter inputs and nutrient availability, thereby reshaping microbial community composition and activity (de Vries et al., 2006; Leff et al., 2015). Changes in plant diversity further cascade belowground, as plant species differ in root exudates and litter chemistry, selecting for distinct microbial assemblages (Steinauer et al., 2016; Wardle et al., 2004). For example, monoculture grasslands dominated by Lolium perenne provide low C:N litter that favours fast-growing microbial groups and reduces overall microbial diversity (Bardgett et al., 1999; de Vries et al., 2006; Liliensiek et al., 2012). Through these pathways, grassland management directly influences the structure of soil communities and the processes they regulate, linking agricultural practice to ecosystem functioning.

Interactions between soil properties (physical, chemical and biological) give rise to soil processes, defined as the physical, chemical and biological transformations that occur within the soil system (Creamer et al., 2022). Organic matter decomposition, mineralisation and respiration are examples of such processes, arising from interactions between soil organisms and their abiotic environment. Multiple processes (e.g. bundles, sensu Creamer et al., 2022) jointly underpin a specific soil function, such as carbon sequestration and nutrient cycling. Through their combined activity, soil organisms form a biogeochemical hub that regulates the fluxes of energy, carbon and nutrients through ecosystems, thereby supporting multiple soil functions simultaneously (Eisenhauer et al., 2026). Within this framework, soil functions represent the mechanistic link between belowground processes and ecosystem services by regulating nutrient cycling, carbon storage and primary production (Bouma, 2014). Closely related concepts such as soil quality and soil health describe the capacity of soils to sustain these functions over time. Soil quality has traditionally been defined as the ability of soil to function within ecosystem boundaries to maintain productivity and environmental integrity (Doran & Parkin, 1994,

1997), whereas soil health emphasises soils as living systems whose functioning supports broader ecological sustainability (Bünemann et al., 2018; Lehmann et al., 2020). Although the terms are sometimes distinguished conceptually (Lehman 2020), they are often used interchangeably in contemporary literature (Bünemann et al., 2018; Creamer et al., 2022). In this thesis, the term soil health is adopted to align with recent policy developments, including the Soil Monitoring Directive and the Soil Health Law (Panagos et al., 2025), while recognising its practical equivalence to soil quality.

The effects of agricultural management on soil communities and functioning

Agricultural practices influence soil functioning by first modifying soil properties and microbial communities, and thereby altering the processes that soils regulate. Fertilisation is a clear example: increased nitrogen inputs reduce fungal biomass and shift microbial community composition, which can diminish nutrient retention and increase nitrogen losses through altered uptake by microbes and plants (de Vries et al., 2006, 2012). As soil functioning declines, agroecosystems become increasingly dependent on external inputs to maintain productivity (Eisenhauer et al., 2011). At the field scale, these effects are rarely driven by single management actions. Instead, farmers apply combinations of fertilisation, biomass removal, and the sowing and steering of the plant community that jointly shape soil chemical conditions and biological interactions. For instance, biomass removal without manure application can decrease soil pH (van Eekeren et al., 2022; van Eekeren et al., 2025), whereas organic manure inputs can buffer acidity and improve nutrient balance (van Eekeren et al., 2009). Such interacting management effects together restructure belowground communities (Tsiafouli et al., 2015) and weaken key biological interactions, ultimately reducing soil functioning (Morriën et al., 2017; Zhang et al., 2020). Re-establishing the natural regulation of soils therefore requires management strategies that rebuild belowground diversity and restore the functions mediated by this diversity.

Methods to measure change

Evaluating whether extensification improves the soil functioning and ecosystem services requires methods that can detect how soil functioning respond to long-term management. Specific functions can be inferred by measuring related soil processes, with both field and laboratory analyses. Because soils rarely deliver a single function in isolation, soil functioning is increasingly assessed in terms of multifunctionality (e.g. multiple soil functions are maintained simultaneously) (Nazaries et al., 2021; Zwetsloot et al., 2022). However,

this poses a methodological challenge: multifunctionality measurements rely on the selection of processes. This selection is often based on time and resource limitation, which limits the number and types of processes that can be measured. This makes aggregated multifunctionality indices sensitive to the choice of underlying processes and how they are combined. As a result, multifunctionality indices may potentially obscure divergent process-level responses (Byrnes et al., 2014; Manning et al., 2018). An alternative and complementary approach is therefore to focus on multiple, mechanistically distinct processes, each underpinning a single soil function. By quantifying several processes associated with nutrient cycling (such as extracellular enzyme activities, organic matter decomposition, and soil respiration) it becomes possible to capture within-function variability and identify divergent responses that are masked by aggregated multifunctionality indices. Such a multi-process, function-focused approach could provide greater mechanistic insight into how soil communities and soil properties jointly regulate soil functioning under realistic field conditions.

A second challenge is to be able to monitor the biological communities that drive them. The ability of soils to perform multiple functions (such as the ability to degrade organic matter and (re)cycle nutrients) depends for a large part on the diversity, abundance and activity of the microbial communities that inhabit them (Chen et al., 2020; Jiang et al., 2019; Maron et al., 2018). Hence, monitoring these communities and their activity is essential to understand whether more extensive management also triggers measurable shifts in microbial community composition that underpin improved soil functioning. Traditional molecular and biochemical approaches, including DNA-based methods and phospholipid fatty acid analysis (PLFA), have greatly advanced our understanding of microbial composition and biomass (Bloem et al., 2004; Fierer et al., 2021). However, these approaches typically capture only a single dimension of microbial systems, such as community structure or potential activity, and are often too costly and time-intensive to serve as practical indicators for frequent monitoring over longer time-periods. Many functional assays are also conducted under controlled laboratory conditions, which may not fully reflect in-situ dynamics (Burns et al., 2013). What remains missing are integrative "fingerprints" that simultaneously reflect microbial composition and metabolic activity under field conditions (Bünemann et al., 2018; Lehman et al., 2015). This motivates the exploration of complementary approaches (e.g. proxies) that can link microbial presence, abundance, and metabolic activity in a more integrative and dynamic manner.

A suitable microbial proxy should be context-aware, measurable over time, informative for decision-making, and deployable beyond research settings (Box 1) (Bünemann et al., 2018; Fierer et al., 2021; Schram et al., 2024). Research suggests that microbial volatile or-

ganic compounds (mVOCs) could be such a proxy for monitoring microbial diversity and metabolic activity (Brown et al., 2021; Insam & Seewald, 2010; Leff & Fierer, 2008). These metabolites, which essentially provide the typical smell to soils, are not only released by plant roots but also by microbes (soil-borne bacteria and fungi) that are present, during secondary metabolism (Garbeva et al., 2014b, 2014a; Tang et al., 2019; Yang et al., 2024). The composition of mVOC profiles can reflect changes in microbial community structure and activity (Abis et al., 2020; Insam & Seewald, 2010; Weisskopf et al., 2021) and could therefore provide insights into how soil communities respond to direct and indirect changes in management intensity. In addition, VOC soil signatures can in principle be detected using commercial sensing platforms such as electronic noses (De Cesare et al., 2011), highlighting their potential for future non-destructive monitoring applications. However, the potential of mVOC profiles to monitor changes in the state of the microbial community has been tested almost exclusively under controlled conditions (Abis et al., 2020; Brown et al., 2021), and mVOCs have not yet been measured in agricultural soils in-situ. Therefore, the potential of using mVOCs as non-destructive, dynamic fingerprints of the microbial community in the field remains to be investigated (Brown et al., 2021). The intensity of agricultural management is known to affect soil microbial communities (Meyer et al., 2013; Romdhane et al., 2022) and hence offer a experimental set-up to measure the changes in microbial community composition, its functioning along a gradient of management, allowing to also study the changes in mVOC profiles.

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Box 1. What constitutes as a suitable microbial proxy?

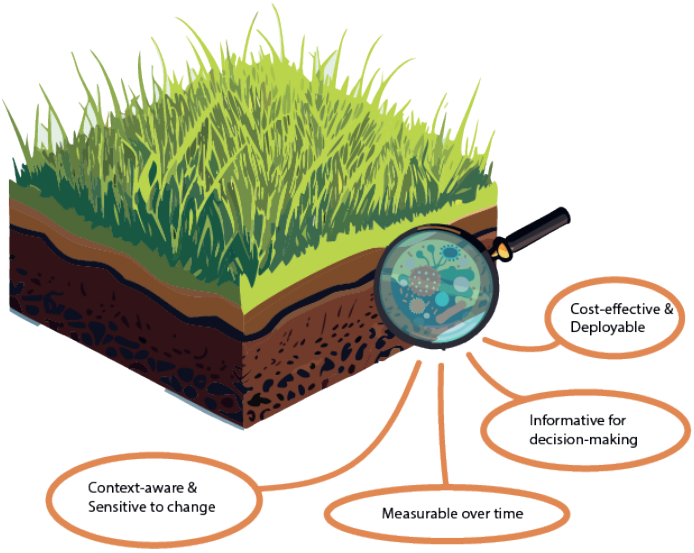


Figure 1.2: Four criteria of an effective soil microbial proxy, based on Fierer et al. (2021), Bünnemann et al., (2018) and Schram et al. (2024).

Context-aware & Sensitive to change: responsive to management-driven shils in microbial community composition or activity, but stable enough that short-term environmental fluctuations do not obscure meaningful biological trends (Fierer et al., 2021). Rather than relying on universal thresholds, microbial indicators should be interpreted relative to appropriate benchmarks or reference conditions, acknowledging that microbial community structure is inherently site- and context-specific (Schram et al., 2024).

Measurable over time: neither so stable that management effects on the microbial community go undetected, nor so temporally variable that repeated sampling yields contradictory results. Microbial indicators operating at seasonal to annual timescales occupy the most useful middle ground (Fierer et al., 2021; Bünemann et al., 2018).

Informative for decision-making: An indicator is only useful if its value can be clearly interpreted and linked to a specific soil function or management outcome (Bünemann et al., 2018). Many existing microbial metrics fail this test, they may detect change without providing actionable guidance (Fierer et al., 2021). Ideally, proxies should link microbial dynamics to processes that land managers can actually respond to.

Cost-effective & Deployable: Methods developed for research setings olen cannot be transferred to routine monitoring or field practice (Bünemann et al., 2018). Practical requirements, including ease of Transitioning to extensive practice sampling, reproducibility, and affordability are essential if proxies are to move beyond the research domain (Fierer et al., 2021; Bünemann et al., 2018)

As soils are known to change slowly when management changes (Heinsoo et al., 2020), an important question is whether this transition can be accelerated. While some studies have reported changes in microbial communities after conversion occurring in 2-4 years (Briar et al., 2007; Verbruggen et al., 2010), others have reported that at least 10 years is needed (Schrama et al., 2018). Historical inputs of fertilisers and pesticides can create legacy effects that persist in soils for decades, slowing the recovery of soil biota and functions (Heinsoo et al., 2020; van Eekeren et al., 2022). High nutrient availability can hinder the establishment of herb-rich vegetation, while the absence of key microbial guilds may limit decomposition and nutrient mineralisation (Schrama et al., 2018; van Rijssel et al., 2022). This phenomenon, which could be defined as a form of soil hysteresis (Beisner et al., 2003), reflects the time lag and path dependence between management change and the transition to a different soil community and functioning. Taking these delayed responses into account is critical for predicting and accelerating ecosystem recovery under extensification and exploring opportunities for accelerating the process of recovery.

Studies have demonstrated that introducing living soil communities or inocula from reference sites, known as soil inoculation, can accelerate the transformation of soil biodiversity and function (Koziol et al., 2019; Wubs et al., 2016). However, the effectiveness of soil inoculation is context-dependent (Gerrits et al., 2023), and the ecological and management conditions under which it successfully enhances restoration outcomes remain poorly understood. In grassland systems, inoculation with soil from herb-rich or semi-natural reference sites has been shown to steer soil microbial communities and plant community composition at "degraded" sites, with donor identity and inoculum amount influencing the trajectories of both belowground and aboveground communities (Han et al., 2022). Soil inoculation may thus help overcome legacy effects and reduce the time required for soils to reach a new functional state following extensification. Testing the potential of soil inoculation for the acceleration of increasing soil functioning in agricultural systems is therefore an important step toward practical soil transformation.

Research objectives

The overall aim of my PhD research was to investigate how the intensity of grassland management affects soil microbial community composition, its metabolic profile (mVOCs), soil properties, and soil functioning, and how these insights can facilitate the transition toward more extensive agricultural systems. Specifically, three key objectives are addressed:

Research questions

1. How does grassland management intensity affect soil microbial community composition, soil properties and soil functioning?
2. Can changes in soil microbial community composition and functioning along these management gradients be detected and monitored using a novel soil microbial proxy based on microbial volatile organic compounds (mVOCs)?
3. Can soil inoculation accelerate the transition from intensively managed to more extensive grassland systems by enhancing soil biodiversity and functioning?

Case study: the Ooijpolder

To study how grassland management is affecting soil communities, properties and soil functioning, I selected permanent grasslands within the Ooijpolder, a riverine agricultural landscape in central-eastern Netherlands, east of the city of Nijmegen. This landscape provides an suitable setting to study long-term management effects, as all fields on the same parent soil types, topography, and climate but differ in management intensity. Agricultural intensification has profoundly reshaped both land use and management in the region (Baumgarten et al., 2025; Nijssen, Remke & Versluis, 2014). During the twentieth century, large-scale land consolidation concentrated farmland within the inner-dyke area and relegated natural habitats to the outer-dyke zones (Baumgarten et al., 2025). This restructuring reduced landscape heterogeneity by removing hedgerows, tree lines, and field boundaries, and also led to an intensification of the dairy farms in the inner-dyke area, resulting in numerous monoculture grasslands with high external inputs. Consequently, the region experienced a loss of aboveground biodiversity and shifts in soil management practices that likely altered belowground community composition and the processes that it delivers.

Transdisciplinary research

This research was embedded within the NWO-funded project Living Lab Ooijpolder-Groesbeek, initiated in 2021 to investigate the process of biodiversity recovery in an intensively managed agricultural landscape. The project brought together researchers from multiple disciplines with farmers, policymakers and other regional stakeholders to jointly explore pathways toward more sustainable land use. By combining long-term ecological research with on-farm experimentation and stakeholder engagement, the Living Lab provides a real-world context in which ecological processes, management practices and societal objectives intersect. This transdisciplinary setting (Roux et al., 2017) shaped both the research questions and the interpretation of results presented in this thesis.

Thesis outline

- **In Chapter 2** I explore traditional and novel approaches to classify grassland management based on farmer surveys from 18 fields in the Ooijpolder, spanning a gradient from conventional high- intensity to semi-natural management. A priori management categories were compared with post- hoc clusters derived from survey data, and both approaches were validated using a remote-sensing derived proxy for land-use intensity.

- **In Chapter 3** I investigate how management intensity affects soil properties, microbial communities (bacteria and fungi), and soil functioning. Focusing on nutrient cycling, I measured three underlying processes, decomposition rate, substrate-induced respiration, and enzymatic activity, following the BIOSIS multiprocess framework.

- **In Chapter 4** I evaluate microbial volatile organic compounds (mVOCs) as potential biomarkers for soil microbial communities. mVOCs were analysed alongside microbial community composition to determine whether specific compounds or profiles could indicate differences in management intensity.

- **In Chapter 5** I test the predictive ability of mVOCs as a classification tool by comparing them to enzymatic activity, using nonlinear support vector classification models.

- **In Chapter 6** I investigate whether soil inoculation can accelerate the conversion of conventionally managed grasslands into herb-rich systems by introducing soil biota from established herb-rich sites.

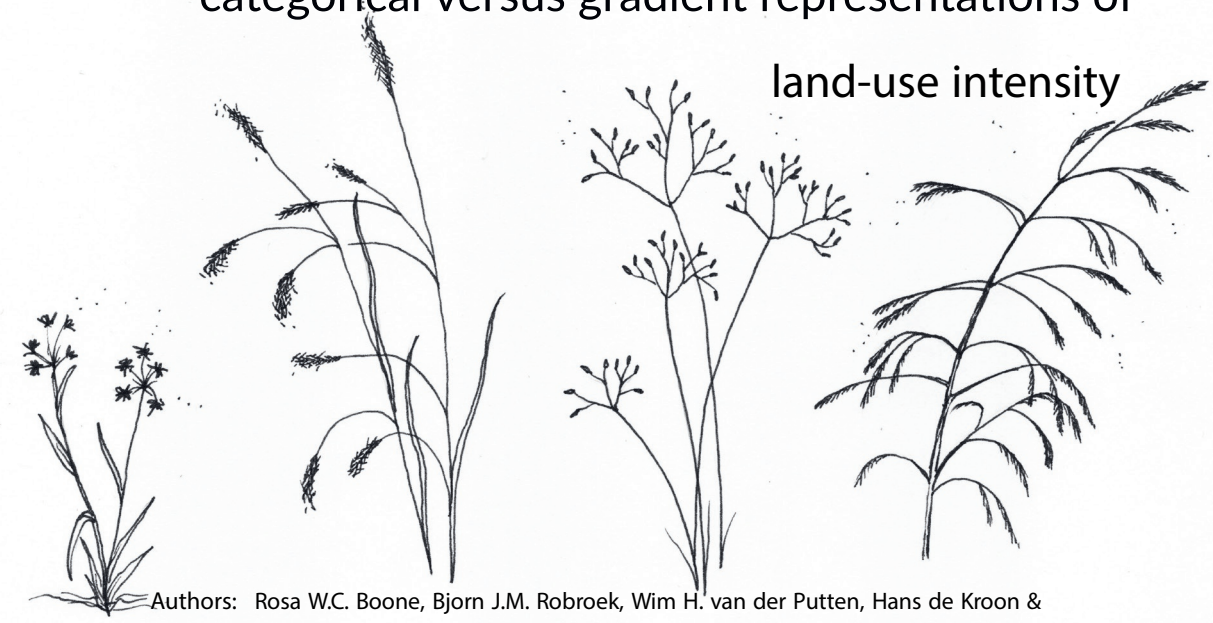
- **In Chapter 7** I synthesise the main findings of this thesis, integrates them with current literature, and discusses implications for farmers, land managers, and policy makers, offering perspectives for future research and sustainable soil management.



Photograph taken at the author's family farm in Zeeland, the Netherlands.

2

A data-driven framework for evaluating categorical versus gradient representations of land-use intensity



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Abstract

Classifying land-use intensity is central to ecological research and soil health monitoring, yet commonly used a priori categories (e.g. "conventional", "extensive", "semi-natural") may oversimplify the diversity of management practices. Such simplification can obscure continuous variation in management intensity that more accurately reflects underlying ecological processes. Here, we present a data-driven analytical workflow to evaluate alternative representations of land-use intensity based solely on management data, prior to linking management to ecological responses. The framework combines unsupervised clustering, gradient-based ordination, and quantitative diagnostics to assess whether management intensity in a given system is better represented as discrete categories, emergent clusters, or continuous gradients. Using management survey data from 18 Dutch grasslands, we compared a priori land-use classes with post hoc clusters and a continuous gradient derived from multivariate management variables. Clustering revealed only weak separation among fields with different category labels, while the gradient captured a smoother pattern of management variation and aligned more closely with an independent remote-sensing proxy. Model comparison further indicated that the gradient-based representation provided a slightly better representation of management intensity than categorical alternatives. Our results demonstrate how data-driven evaluation of classification structure can inform the choice of representation for land-use intensity, reducing the risk of imposing arbitrary categories on continuous management variation. The proposed workflow is broadly applicable to ecological contexts where heterogeneous management or monitoring data must be translated into accurate variables, including environmental monitoring and ecosystem assessment.

2.1 Introduction

Land-use intensification is a key driver of ecological degradation, with strong and well-documented effects on both above- and belowground biodiversity (Le Provost et al. 2021). Intensified agricultural land use alters soil physical, chemical and biological properties, often reducing functions such as nutrient cycling, water retention, carbon sequestration and habitat for biodiversity (Kopittke et al. 2019). These changes have caused widespread concern over the sustainability of intensive agriculture and have spurred interest in soil monitoring to improve soil health, which is increasingly important for soil health monitoring programs and emerging legislative frameworks such as the EU Soil Monitoring Law (the first EU legislation on soils).

A central challenge in this effort is to determine how to classify the intensity of management and relate it to ecosystem processes (Shennan et al. 2017; van Rijssel et al. 2025; Blüthgen et al. 2012; Giller et al. 1997). Historically, categorical labels (e.g., 'conventional', 'organic', 'extensive') have been used to capture the variation in intensity of land management in agriculture. Although simple and easy to interpret for a wide audience, a priori categorization may mask variability within categories and, as a result, may reduce the discriminative power of comparing effects of land management and land-use intensity (Shennan et al. 2017; Puech et al. 2014; Sumberg and Giller 2022). The question is: how can we adequately represent differences in land-use intensity in experimental designs?

A priori categorization is widely used in field-based soil studies, where management categories are assigned prior to data collection (Boinot et al. 2025; Vries 2012; van Dobben et al. 2019). In some cases, such as organic versus conventional, organic farm management is prescribed by certification, such as Stichting Keur Alternatief voortgebrachte Landbouwproducten (Biocontrole 2021) in the Netherlands. There is no such certification for conventional, regenerative, or other farming systems. Moreover, certain aspects, such as the intensity of land use, may apply to both conventional and organic farming (Rijssel et al. 2025). Therefore, even when certified, a priori categories may obscure differences in land management and their effects on ecosystem functions (Sumberg and Giller 2022; Shennan et al. 2017). Because categories are often applied without post priori verification with data on actual practices, they may group together fields with quite different land use intensities. For instance, some organic-certified fields may still be intensively used with implication to soil multifunctionality (Rijssel et al. 2025). In addition, the term 'conventional' has become ideologically charged, obscuring the diversity of practices it encompasses (Sumberg and Giller 2022). These issues highlight the need to critically evaluate a priori classifications and consider data-driven alternatives when assessing land-use impacts.

To overcome limitations of a priori classifications, it has increasingly been advocated to use continuous gradients or management indices to capture variations in land-use intensity (Giller et al. 1997; Armengot et al. 2011; Blüthgen et al. 2012). Non-a priori classifying approaches quantify intensity based on multiple variables, such as fertilizer input, mowing frequency, grazing pressure, and livestock density. By aggregating these variables, gradient-based approaches provide a more nuanced view of land management than binary or ternary labels. Studies by Blüthgen et al. (2012) and van Rijssel et al. (2025), for example, have shown that post priori intensity indices could better predict soil multifunctionality, and other functional outcomes in their datasets, than a priori categorical labels could do. However, variable selection, weighting schemes, and regional specificity all influence index performance (Armengot et al. 2011). Despite these potential caveats, gradients offer a promising alternative to traditional labels, especially when combined with multivariate methods such as principal component analysis (PCA) that allow for objective data reduction and pattern recognition.

Although there is a growing interest in gradient-based and hybrid approaches, few studies are available that determine when continuous indices are a better fit than categorical labels. Ideally, this decision should be made prior to ecological data collection, yet in practice, approaches often rely on post-hoc assessments by testing whether observed ecological patterns align better with discrete groups or continuous gradients. This post-hoc data approach, however, carries the risk of bias, as it may favour the structure that best fits the expected outcome. To avoid bias, objective, data-driven methods are needed to assess the validity of classification schemes based solely on management information, before analysing ecological responses. However, currently there is no clear data-driven basis for deciding whether management is best represented by discrete categories, post hoc clusters, or continuous gradients. Clustering techniques, such as hierarchical clustering using Ward's method (Murtagh & Legendre 2014), allow for post priori clustering based on empirical management data, which can be directly compared to a priori categories. Evaluation metrics such as the silhouette width and the Adjusted Rand Index (ARI) quantify the degree of alignment between these schemes (Alippi et al. 2009; Lengyel & Botta-Dukát 2019).

Conversely, if no natural clusters are found, this may justify the use of gradient-based models, although their sensitivity to input variables also requires careful evaluation. This raises a practical question: how can we decide, using only management data, which classification approach is most appropriate for a given dataset?

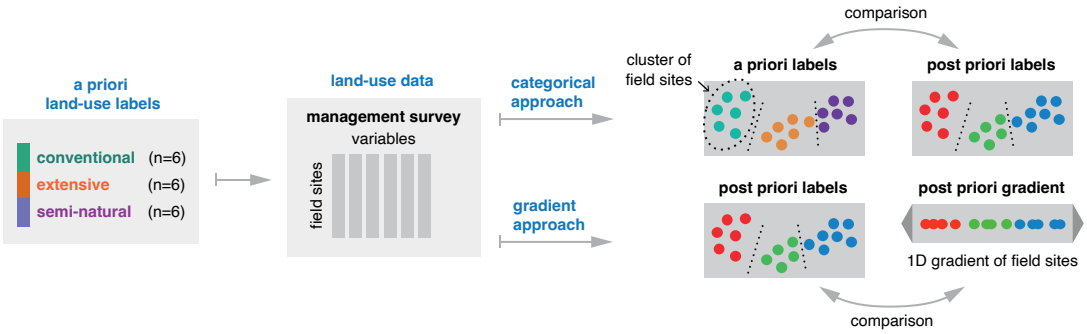


Figure 2.1: Overview of workflow for comparing a priori categories with post priori clusters and gradient of land use.

Here, we present a data-driven analytical framework that can be applied to any management dataset to evaluate the structure of land-use intensity before ecological analyses are conducted. Specifically, we (1) compare a priori categories with post priori clusters derived from farmer surveys, and (2) compare a- and post priori clusters with a gradient approach (an overview of the method is shown in Fig. 2.1). We focus on evaluating whether different classification strategies reveal consistent structure within management practices alone, without reference to their ecological consequences. However, in order to validate the classification approach, we use a remote-sensing-derived proxy for grassland management intensity. Remote sensing provides an independent means of validating grassland management intensity (Ali et al. 2016).

Recent advances in spatial, temporal and spectral resolution now allow parcel-level discrimination of management practices (Bekkema and Eleveld., 2018). In particular, the Sentinel-2 Red Edge Position (S2REP), sensitive to vegetation type, chlorophyll concentration and leaf nitrogen content (Frampton et al. 2013), offers an objective, spatially consistent proxy for grassland management intensity that does not rely on farmer self-reporting surveys. Incorporating such a proxy enables us to test whether our classification schemes capture differences on the ground. With S2REP as a validation, we tested the hypothesis that post priori clusters (derived from actual management data) would differ from a priori categories, and account for a greater share of the observed variation in grassland management intensity. Furthermore, we tested the hypothesis that a gradient-based approach would explain most of the variation in grassland management intensity, as compared to a priori categories and post priori clusters, using 18 Dutch grasslands as a demonstration case.

2.2 Methods

2.2.1 Regional context of the case study

The data for this study was collected from the Ooijpolder (51°51'53.9"N, 5°54'36.0"E), The Netherlands. The Ooijpolder is situated between the river Waal (North) and the city of Nijmegen (West), close to the border of Germany. The area has a long history of agriculture (mainly dairy and orchards) and is nationally known as an exemplary case for nature and landscape development. Similar to national developments, in the second half of the 20th century, the area underwent an intensification of agriculture, which has been impacting local flora and fauna. However, due to recent restoration efforts, biodiversity is improving again (Baumgarten et al. 2025; Nijssen, Remke, and Versluijs 2014). A priori selection and post priori management information Here, "a priori" refers to predefined management categories based on field information, whereas clustering was performed in an unsupervised, non-Bayesian manner to derive data-driven ("post priori") groups.

The study was conducted using 18 grasslands in the Ooijpolder, within the same sites described in (Boone et al. 2025). In September 2022, grasslands were selected in collaboration with farmers and landowners and categorized into three a priori land-use intensity classes (shown in Fig. 2.2, details are described in table S2.1):

1. **onventional**: perennial ryegrass (Lolium perenne L.) monocultures receiving chemical inputs (fertilizers and pesticides);
2. **extensive**: herb-rich swards with no pesticides and only organic manure applications;
3. **semi-natural**: low-productive, herb-rich grasslands with no external manure inputs.

Each category included six fields that served as independent replicates (n = 6). All fields have been under consistent management for at least 10 years. For each field, we conducted management surveys with farmers to gather information on the general management practices applied over the past 10 years (details on practices are described in table S2.2). Management surveys consisted of questionnaires about the following categories: history of management, fertilization, mowing and grazing regime and pesticide usage. Additionally, to retrieve data about the plant richness, in each field, three randomly located 2 m × 2 m plots were used for vegetation surveys in June 2023, using the Braun-Blanquet method (Westhoff 1973).

To validate our post priori land use analysis, we calculated a remote-sensing-derived proxy for grassland management intensity (Bekkema and Eleveld 2018): Sentinel-2 Red-Edge Position (S2REP). Recently, (Bekkema and Eleveld 2018) demonstrated that S2REP was able to classify management intensity of Dutch Peatlands to a high accuracy. Although soil type showed to have a significant impact on S2REP values, this should not be of influence for our dataset, because the soil type in our study is comparable (bodemdata.nl).



Figure 2.2: Examples of a priori grassland classes from photos taken in April 2024: conventional, extensive and semi-natural

Sentinel-2 data was extracted from Google Earth Engine, for the period between 01-04-2022 and 30-04-2022, using images with a minimum of 10 percent clouds. S2REP values were calculated using the following formula:

$$'705 + 35 * (((B6 + B4)/2 - B5)/(B6 - B5))$$

where B4, B5 and B6 are surface reflectance values from Sentinel-2 bands centered at 665 nm (red), 705 nm (red-edge), and 740 nm (red-edge), respectively. After visual inspection, only the dates with the most stable values were selected for extraction. For the composite image, the mean of two dates were calculated and the image was exported as a .tif file. One conventional field was not covered by the composite image due to cloud coverage. Therefore, we removed one conventional field from the subsequent analysis. To obtain S2REP values for each field, we used the center points of the 2 m × 2 m vegetation survey plots, resulting in three values per grassland. Plot center points were digitized in QGIS as a vector layer and exported as coordinate data. In R, using the raster, terra, and exactextractr packages, we aligned the coordinate file and the S2REP median raster (.tif) to the same CRS (EPSG:28992, RD New Amersfoort). A 2m radius buffer was applied around each plot center, and the median S2REP values within each buffer were extracted.

2.2.2 Data-driven analytical framework

For variable selection, scaling and clustering, we used R Studio version 2024.09.1+394 (Posit team 2024).

Selecting management variables

To identify post priori management clusters, we used a clustering method. For this, management variables were first selected from the farmer survey data. We made sure to only select variables that were suitable for clustering. Hence, either continuous numerical or binary (1 = yes, 0 = no) variables were selected. Spearman correlation analysis was used to assess multicollinearity (threshold: Spearman R > 0.7). Three highly correlated (R > 0.8) but ecologically informative variables (plant species richness, chemical manure input (kg N/ha/year), and organic manure input (m^3/ha/year)) were retained to preserve management diversity (Fig. S2.1). Although this threshold is higher than the typical R > 0.7 (Dormann et al. 2013), high levels of multicollinearity were accepted, as interdependence among farming practices is typical (for example, fertilization often co-occurs with frequent mowing (Armengot et al. 2011). For instance, conventional farmers used both chemical and organic manure, whereas extensive farmers applied only organic manure.

Retaining both allowed for greater within- and between-group variation. The selection resulted in eight management variables (listed in table S2.2).

Variable standardization

To create post priori clusters from the farmers' survey data, all selected management variables were standardized using z-score transformation. Despite the non-Gaussian distribution of some variables and the known sensitivity of standard scaling to outliers, this approach was chosen to preserve meaningful variation across sites (e.g., chemical fertilization was applied only in conventional fields, creating high-value contrasts, or outliers).

Clustering method

For clustering, the first step was a systematic and quantitative process of model selection, by which a clustering method was selected from among multiple and diverse candidates, based on achieving the best cluster separation on the data (see Supplementary Information 2.A). The clustering model selected was hierarchical clustering using Ward's minimum variance. For this, an Euclidean distance matrix was first computed. This was followed by applying hierarchical clustering using Ward's minimum variance (ward.D2, (Murtagh & Legendre 2014)). The number of clusters was set a priori at k = 3 to match the original a priori categories.

With the distance matrix created from the standardised data, Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP, using the umap package in R) were performed to visualise the distributions in two dimensions, and to allow for comparison of a priori and post priori clusters. The same visualization could be done with PCA, but the advantage of the nonlinear UMAP over PCA specifically is that a UMAP visualization can separate samples even when they overlap (because their coordinates are nearly identical). This overlap happens frequently among the 3 samples from the same field, which are naturally identical in many management variables. A minimum distance in the plot coordinates is placed in UMAP, such that all samples are clearly visible in the plot. The UMAP parameters used are: n_neighbors = 17 (such that the number of neighbours of a field sample, together with the field sample itself, match the average cluster size of 18), and min_dist = 0.12.

In the two-dimensional visualization, the fields were color-coded according to their respective post priori cluster assignment using ggplot2. As a statistical measure of comparison between the clusters (a priori vs. post priori), we calculated the Adjusted Rand Index (ARI) (Hubert and Arabie 1985) using the MixGHD package (Tortora et al. 2021). ARI mea-

sures the similarity of the two sets of labels over the same points, by counting the pairs of points that are assigned in the same (or different) clusters in the two sets of labels, adjusted for chance. ARI ranges in the interval [-1,1], with values close to 1 meaning very good consensus between the two sets of labels, and values around 0 when the labeling is close to random (independently of the number of clusters and samples).

Clustering performance was evaluated using average silhouette width (calculated via the cluster.stats function from the fpc package). This metric shows how well the clusters are separated in terms of the distances between data points. The metric ranges in the interval [-1,1], with values close to 1 meaning very good cluster separation, and values near 0 meaning that the clusters overlap. We also inspected the cluster sizes, namely whether these are balanced. Additionally, we used the Gap Statistic, which compared within-cluster dispersion to that of a null reference distribution, and provides the optimal number of clusters within a dataset. For this, we used clusGap function within the mclust package, with K.max = 10 and B = 500.

Gradient method

To both extract and visualize a gradient from the post priori data, we used a linear Sparse Principle Component Analysis (Sparse PCA) analysis (Erichson et al. 2020). Sparse PCA finds the set of sparse principal components that can optimally reconstruct the data, such that some of the coefficients are zero. The amount of sparseness is controllable: we configure the method to the ideal sparsity, namely that each management variable appears (on average) only once among the sparse principal components. Compared with PCA, Sparse PCA has similar or slightly lower total explained variance (here, approx. 3% lower in two dimensions), and does not ensure complete orthogonality among multiple principal components. However, it gains in the interpretability of the principal components. In R, we used the sparsepca package (spca function) to compute Sparse PCA (Erichson et al. 2020). We first extracted the variable contributions for both PC axes, to assess possible unbalanced contributions. As gradient, we use the first PC axis (58.15% explained variance), and also show the first two PCs (75.15% explained variance) as a two-dimensional gradient.

Evaluation method

To evaluate the three classification approaches (a priori categories, post priori clusters, and the continuous gradient), we fitted linear mixed-effects models with S2REP as the response, each classification scheme as the fixed effect, and Field as a random intercept. We used linear mixed-effect models in order to account for the hierarchical structure and site-

level heterogeneity (Boone et al. 2025). Residual heteroscedasticity was assessed using the simulateResiduals function in the DHARMa package. When residuals deviated from normality, we instead fitted a generalized mixed-effects model (family = Gamma, link = log) using glmmTMB. Results from the LMMs were visualized with the emmeans package, applying pairwise comparisons among categories. In addition, we quantified the proportion of variance explained by each model using conditional R^2 as implemented in the MuMIn package. Model performance was additionally compared using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). These criteria provide relative measures of parsimony within the candidate model set, balancing goodness-of-fit against model complexity. They do not quantify explanatory power in an absolute sense, but allow ranking of alternative model representations. Lower values indicate comparatively more parsimonious models.

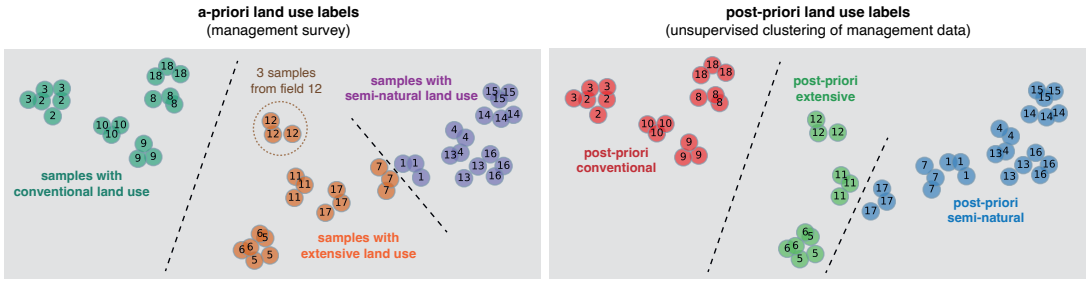


Figure 2.3: Comparison of a priori categories and post priori land-use clusters. UMAP maps of plots coloured by a priori categories or post priori clusters. The labels are similar but not identical (ARI = 0.72). Each plot is labelled by the field identifier (from 1 to 18), with 3 plots per field.

2.3 Results

2.3.1 A priori versus post priori clustering

Post priori clustering of the management variables yielded groupings that were broadly comparable, though not identical to the a priori categories (ARI = 0.72). Visual comparison of the a priori categories and post priori clusters (Fig. 2.3) revealed a shift in the distribution of the field plots, with two a priori extensively managed sites reassigned to the semi-natural cluster in the post priori categorisation. This shift resulted in an imbalance relative to the previously equal distribution of plots across categories (conventional n = 6, extensive n = 4, semi-natural n= 8).

Overall, the post priori clusters exhibited weak separation, as indicated by an average silhouette score of 0.340. Silhouette scores close to 1 indicate well-separated clusters, and scores below 0.5 are generally considered indicative of poor clustering structure

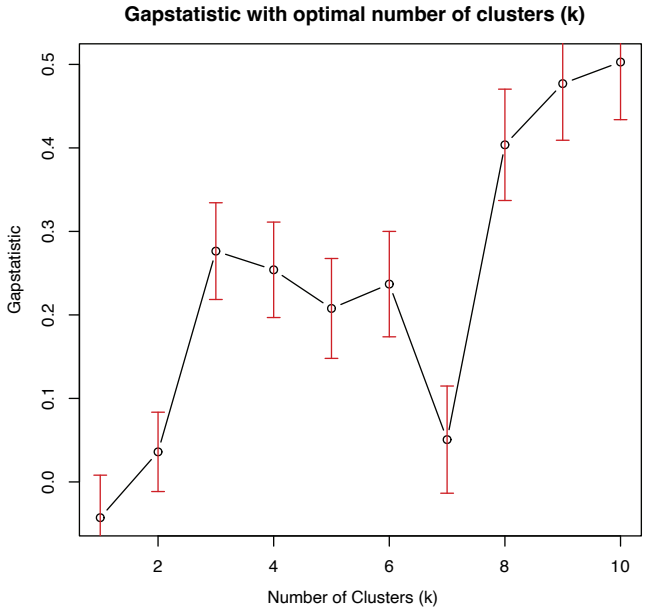


Figure 2.4: Gap Statistic values (mean ± standard error) for different numbers of clusters (k) based on management variables. Higher values indicate better separation between clusters. The continued increase of the Gap Statistic indicates that no optimum is reached with k = 10.

(Rousseeuw 1987). Supporting the low amount of clustering, the Gap Statistic showed no clear optimum number of clusters k, with the gap continuing to increase up to k = 10 (Fig. 2.4). This suggests the absence of well-separated clusters and instead supports a continuous gradient.

2.3.2 Gradient of land-use intensification

The plots gradually aligned along the first PC axis (Fig. 2.5 shows the one-dimensional gradient), which explained the majority of the variation (58.15%). All variables were shown to contribute roughly equally to the first PC axis, except for the Age of grassland, which showed to relate solely and most strongly to PC2 (Fig. S2.2) shows the two-dimensional gradient). All variables seem to cause variability within post priori clusters.

Along the one-dimensional gradient, the fields ranged from most intensive practices (conventional grasslands) to least extensive practices (semi-natural grasslands). Organic manure input (m^3 ha-1), plant species richness, and nitrogen input by chemical fertilizer (kg N ha-1) contributed most to the distribution (each having linear coefficients with abso-

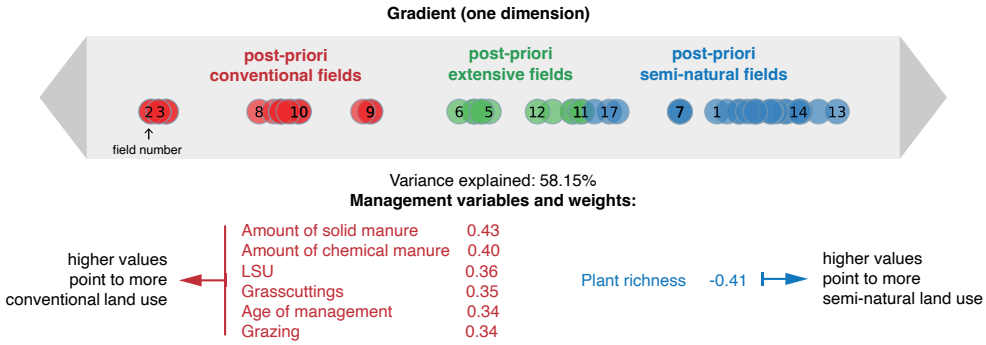


Figure 2.5: One-dimensional gradient for land-use intensity. The plots are identified by their field number and coloured by their post priori land-use label like in Fig. 3, but only some field numbers are shown for clarity. The subset of management variables which define this gradient are shown at the bottom, with (and sorted by) their linear coefficients (higher values for management variables with positive coefficients point towards the conventional category of management, and higher values for those with negative coefficients point towards the semi-natural category).

lute values above 0.40).

2.3.3 Evaluation

All three classification approaches (a priori categories, post priori clusters, and gradient) explained substantial variation in grassland management intensity (S2REP values), as in Fig. 6. A priori categories, post priori clusters and the gradient each explained roughly 96% of the variance in grassland management intensity values after accounting for site effects (conditional R^2=0.956, 0.955 and 0.955, respectively). Linear mixed-effects models with Field as a random factor were highly significant predictors of grassland management intensity (a priori: $F_{2,17}$ = 27.8, p < 0.001; post priori clusters: $F_{2,17}$ = 28.06, p < 0.001; gradient: $F_{1,18}$ = 53.94, p < 0.001). Pairwise comparisons indicated that all categories within both a priori categories and post priori clusters differed significantly from each other (p < 0.05).

Model comparison metrics (Fig. 2.6) showed slightly lower AIC and BIC values for the gradient model (AIC = 95.23, BIC = 102.96), indicating marginally greater parsimony relative to the categorical alternatives. However, differences among models were small (post priori clusters: AIC = 97.69, BIC = 107.35 and a priori categories models: AIC = 97.80, BIC = 107.47).

2.4 Discussion

2.4.1 A priori vs post priori

As hypothesized, despite some overall agreement, the match between the a priori categories and post priori clusters was imperfect, indicating that certain variation was overlooked by the a priori categories. In practical terms, several fields initially labelled 'extensive' ended up clustering closer to 'semi-natural' group, when using the management variables. Interestingly, both fields are rented by conventional farmers from national forestry services, and are therefore managed with considerable lower inputs and without pesticides, as compared to their conventional grasslands of those farmers. Although these formerly labelled 'extensive' grasslands are still used as production grasslands for farmers, the management was even less intensive than the generic 'extensive' label implied. This shows that a simple category assignment, based on farmers' perceptions, can overlook such variation. Moreover, the post priori clusters yielded slightly lower AIC and BIC values, although the differences were minor. Thus, despite these differences, both approaches demonstrated broadly comparable explanatory power. Although we did not explicitly investigate the underlying causes, mismatches between a priori-defined categories and post priori clusters may be related to the 'cultural repertoires' of conventional farmers, whose production-oriented perception of farming (Maaten 2009; De Snoo et al. 2013) shapes how they classify the intensity of management.

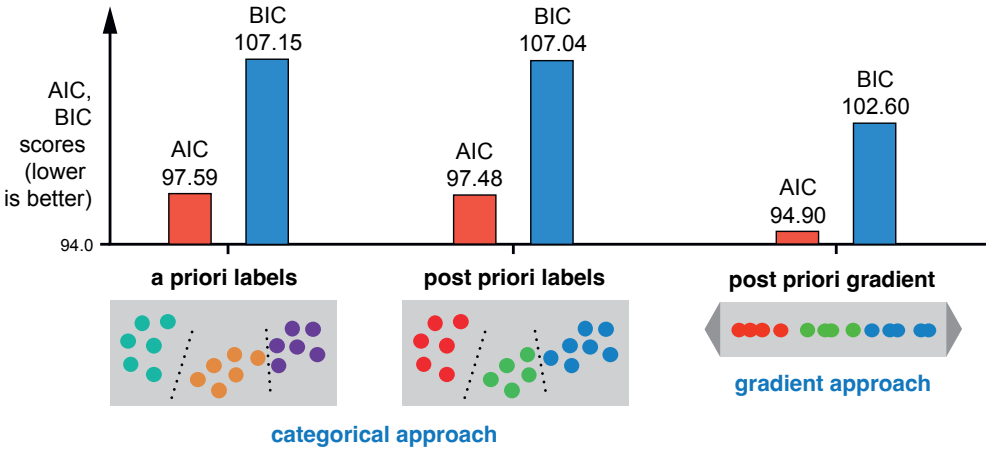


Figure 2.6: Comparison of categorical and gradient classification approaches. 1) In the categorical approach, data were grouped according to either a priori field labels or post priori labels, yielding similar model fit (in AIC and BIC scores). 2) In the gradient approach, fields were arranged along a continuous post-priori gradient, which resulted in a lower AIC and BIC, indicating an improved model fit compared to categorical classifications.

Beyond this conceptual lens, a priori assignment of a field to a farming category does not necessarily reflect the actual management carried out within that category, but can have strong consequences for biodiversity (McCracken et al. 2015). For example, McCracken et al. (2015) showed that the effectiveness of flower strips for biodiversity depended not only on environmental conditions but also on the farmers' experience and interpretation of agri-environmental schemes. Similarly, while a farmer may categorize a field within a certain management class a priori, the way that field is ultimately managed may deviate from the generic label, with important implications for biodiversity (van Rijssel et al. 2025). These findings underline the need for objective, data-driven assessment of management intensity when fields are used in ecological studies or environmental monitoring.

2.4.2 Land-use as a gradient: a stronger alternative to clustering

In our study, the analyses indicate that land-use intensity is more appropriately conceptualized as a continuum, supporting our second hypothesis. Although variation in grassland management intensity (S2REP) was explained to a similar extent across all three models (conditional R^2 = 0.956, 0.955 and 0.955, respectively), the low silhouette score indicating weak internal cohesion, together with slightly lower information-criterion values (AIC/BIC) for the gradient model, suggested marginally greater parsimony relative to the categorical alternatives. However, because information criteria only rank models within the candidate set, we interpret these differences cautiously and primarily in combination with the structural diagnostics (silhouette width, gap statistic, and ARI) when assessing whether management intensity is better represented as clusters or gradients. In practical terms, the fields labelled as different categories were often nearly as similar to each other as to those within their own group, meaning that clear group boundaries are lacking. This impression was reinforced by the gap statistic: It did not identify any clear 'optimal' number of clusters in the management data, implying an absence of a well-separated group structure.

Instead of a tidy partition into distinct categories, the management intensity appears to grade continuously from lowest to highest across fields along the first PC axis. The extracted gradient represented the relative position of the fields to one another based on the management practices applied over the past 10 years. This outcome is consistent with observations from other agricultural studies that caution against binary classifications. Puech et al. (2014) observed a wide diversity of farming practices distributed along a continuum from organic to conventional systems. Similarly, Armengot et al. (2011) reported substantial overlap within this gradient, with some fields from different categories shar-

ing the same LUI index values. More recently, van Rijssel et al. (2025) demonstrated that a continuous metric of management intensity provided greater explanatory power than a simple conventional-organic dichotomy.

Our findings support the recommendations of Blüthgen et al. (2012) for data-driven, multidimensional indices of land-use intensity based on actual input data rather than assumed categories. Their work in grassland systems emphasized the variability within categories and the predictive strength of composite indices. However, their work was based on an index created by summing three standardized compounds (mowing, grazing, and fertilization). Specifically, they refrained from alternatives such as ordination techniques because of the notion that it can create uninterpretable gradients when variables have complex relationships. In our approach, we chose for a PCA-based gradient, sensu (Armengot et al. 2011), because we did not want to restrict ourselves to only mowing, grazing, and fertilization. We also wanted to include temporal variables such as Age of grassland and Age of management, because it is known that temporal factors can also influence the intensity of management (e.g. 'soil hysteresis'), which can have an effect on ecosystem processes (Morriën et al. 2017; Squire et al. 2015). For example, (Rijssel et al. 2025) showed that fungal communities become more dissimilar with time since conversion from conventional to organic agriculture. However, our data set only looked at how long the same management has been applied for all fields, and not the 'time since conversion' from conventional to extensive or semi-natural management.

In the future, 'time since conversion' would be an essential variable to add, although information on historical management is sometimes difficult to retrieve, and it is not known to what extent it should go back in time. Additionally, we also included plant richness as an input variable of management intensity. Plant richness is often thought of as an output variable, e.g., where farmers management influences the richness of plant species that occur on their fields. In Blüthgen et al. (2012), they used the Ellenberg nitrogen indicator value as a response variable to correlate their index with the plant community. However, in our approach, we use plant richness as an input variable, because farmers directly manage plant communities on their fields, by using herbicides, weeding, or sowing seed mixtures. Therefore, we used plant species richness as a management variable. When considering its contribution to the gradient, it was the only variable that had a negative effect on the direction of the distribution but was shown to be equally important as the other variables.

Although our gradient has been tailored to our study system, our analysis explicitly provided tools to test whether the data supported a gradient approach, using the Gap Statistic, silhouette width and ARI. These methods can help researchers in the future to avoid assuming gradients where none exists or imposing categories when a continuum better rep-

resents the variability in management intensity. This is particularly relevant for environmental monitoring schemes that require consistent classification of management intensity across heterogeneous landscapes. These findings highlight that classification choices should be based on empirical management information prior to analysing ecological outcomes, as relying solely on predefined labels may obscure meaningful environmental variation.

Despite these strengths, several limitations should be acknowledged. Our results are based on a relatively small number of fields and a geographically restricted study area within a small country, which may limit the generalisability of the specific quantitative patterns observed. Consequently, the exact structure of management gradients or clustering outcomes may differ in larger or more heterogeneous regions. However, the analytical framework itself is not constrained by sample size or geography. Because it relies on standardized, data-driven multivariate methods, it can be readily applied to datasets of any scale and to agricultural systems in other regions. Thus, while the empirical findings should be interpreted within the context of our study system, the methodological approach provides a broadly transferable tool for assessing management intensity across diverse landscapes.

2.5 Conclusion

As Blüthgen et al. (2012) already pointed out, "quantifying land-use intensity is not a trivial issue". As there are many different ways of quantifying land use intensity, broad categorizations (such as 'conventional' vs. 'organic') could fail to capture the variability present in real farming systems, limiting our understanding and ability to study the effect of land use intensity on ecosystem processes. We treated land-use intensity as a continuous gradient, leading to improved capturing of the variability in the data compared to categorizations. Moreover, even when using the predefined categories, there was not a perfect match with post priori labelling. In general, these results demonstrate that all three classification approaches capture a strong and highly significant structure in grassland management intensity, with the continuous gradient offering a slightly more parsimonious fit.

Ecologists, land managers, and monitoring programs are increasingly facing the challenge of classifying heterogeneous land use systems. This paper has provided easy-to-use metrics that can aid researchers and practitioners in selecting the best approach for their data, using only management data and freely available remote sensing data. Our results support a shift away from a priori categorical categories toward continuous, empirically derived gradients that reflect actual management. This approach enables more nuanced comparisons across studies, helps avoid confirmation bias, and provides a stronger foundation to link land use intensity to ecological outcomes. These results advocate the use of a gradient-based classification, supported by transparent metrics and visualizations, as a default approach unless categorical distinctions are clearly justified.

2.6 Acknowledgements

We would like to acknowledge Nick van Eekeren from the Louis Bolk Institute for having a significant contribution in the process of selecting the grasslands. We also would like to thank the farmers for their time and the provision of data and for allowing us to use their fields as a research object. Funding for DB was provided by the SoilPros project from the Netherlands Institute of Ecology (NIOO-KNAW). RB was funded by imec, through the OnePlanet Research Center (The Netherlands).

2.7 Data availability statement

Data available from the DANSEasy Digital Repository https://doi.org/10.17026/LS/J4Y6FO (Boone & Bucur, 2026).

Chapter appendix

2.A Supplementary information

2.A.1 Clustering method: model selection

For model selection, we first selected a set of candidate clustering methods, with the objective that this selection covers a variety of techniques. The candidate methods were:

1. k-means clustering with k-means++ initialization (Forgy, 1965),
2. mean shift clustering (Comaniciu & Meer, 2002),
3. four variants of hierarchical clustering using different linkage types for merging clusters successively: Ward, complete, average, and single (Murtagh & Contreras, 2017),
4. DBSCAN (Ester et al., 1996), and
5. HDBSCAN (Campello, Moulavi, & Sander, 2013).

When the parameters of these methods included the number of desired clusters in the output, this was set to 3, which is the number of a priori clusters. Other important parameters were tuned, by linearly searching for the optimal value within a range.

All candidate methods were applied to the management data, and their ability to separate clusters was measured (in unsupervised fashion, so without any ground truth). Multiple measurements were used: the Caliński-Harabasz index (Caliński & Harabasz, 1974), the Davies-Bouldin index (Davies & Bouldin, 2009), and the silhouette score (Lengyel & Botta-Dukát, 2019). Consistently, the hierarchical clustering with Ward linkage produced the best performance.

2.B Supplementary figures

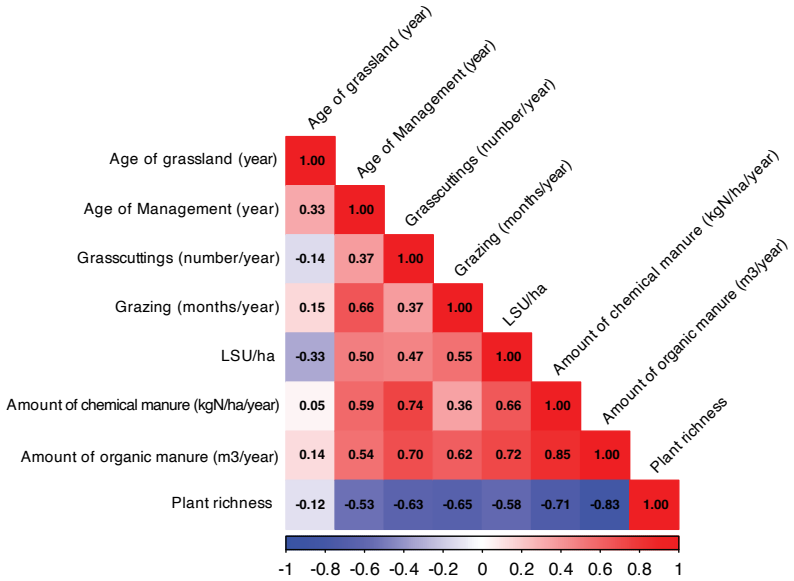


Figure S2.1: Correlation matrix showing pairwise Spearman's ρ values between all selected management variables (colour scale from -1 to +1).Correlation matrix showing pairwise Spearman's ρ values between all selected management variables (colour scale from -1 to +1).

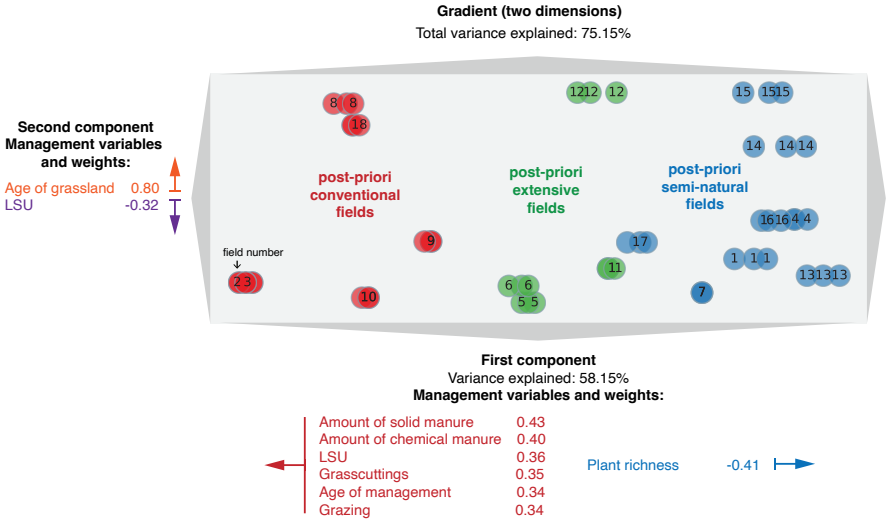


Figure S2.2: Two-dimensional gradient for land-use intensity. The plots are identified by their field number and coloured by their post priori land-use label like in Fig. 3. The subset of management variables which define this gradient are shown at the left and bottom sides, with (and sorted by) their linear coefficients.

2.C Supplementary tables

Table S2.1: Selected management variables and observed values across study fields as described in Boone et al., 2025.

Type	Age grassl. (yr)	Age mgmt (yr)	Cuttings /yr	Grazing (mo)	LSU /ha	Chem. fert.	Manure (m^3)	Pest. use	Plant cover
Conventional	86	39	3	8.0	3.0	Yes	44	Yes	English perennial ryegrass
Extensive	56	21	1.5	6.8	2.3	No	17	No	Herb-rich swards
Semi-natural	81	17	0.5	0.0	1.0	No	0	No	Nature-inclusive swards

Table S2.2: List of selected management variables used in the analysis, including their description and observed range across study fields.

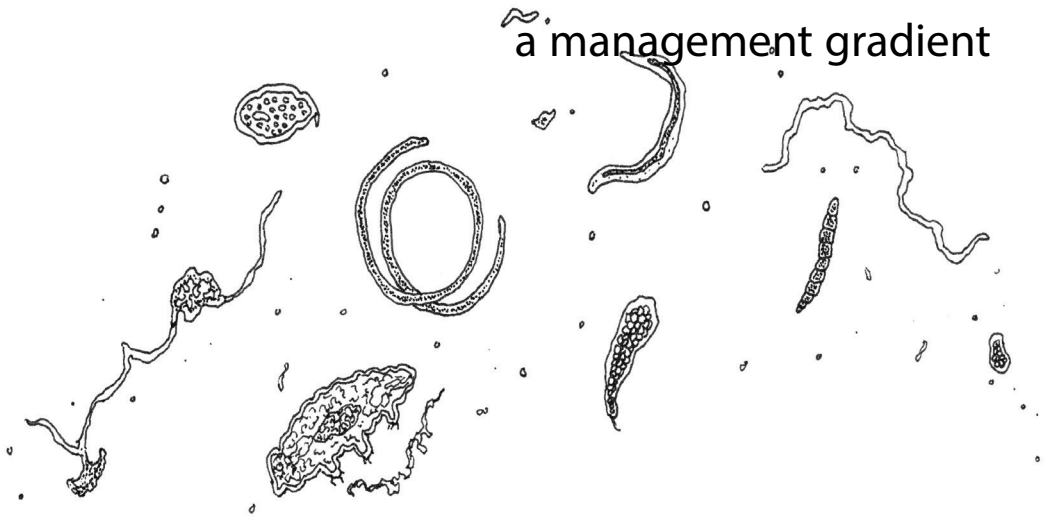
Number	Variable	Description	Range
1	Age of grassland (year)	The number of years it has been a grassland	15-173
2	Age of management (year)	The number of years it has been managed under the current management regime	10-57
3	Grasscuttings (number/year)	The number of grasscutting events per year	0-5
4	Grazing (months/year)	The number of months the grassland is grazed by cows	0-10
5	LSU/ha	Unit of Livestock per hectare	1-5
6	Amount of chemical manure (kg N/ha)	The amount of chemical fertilizer in kg Nitrogen per hectare per year	0-175
7	Amount of organic manure (m^3/ha)	The amount of organic manure in cubic meters per hectare per year	0-45
8	Plant richness (nr. of species)	The number of individual species found in each plot (2x2 m)	2-22



Photograph taken at the author's family farm in Zeeland, the Netherlands.

3

Variations in soil functioning of grasslands along a management gradient



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Abstract

Agriculture in Europe is moving toward greater sustainability along two main paths: continued intensification supported by new technologies, and an increasing shift toward less intensive approaches. The latter is expected to improve soil abiotic and biotic properties and, thereby enhancing soil functioning. Yet the underlying mechanisms remain poorly understood under on-farm conditions. We examined how management intensity influences soil abiotic attributes, microbial communities, and processes related to nutrient cycling along a locally constrained gradient of permanent grasslands (within a 12-km radius). Farmer surveys on management practices were combined with measurements of soil organic carbon (SOC), pH, bulk density, nitrogen, bacterial and fungal communities, and three complementary indicators of organic matter degradation: litter decomposition, extracellular enzymatic activity, and evenness of substrate utilisation). Management intensity significantly altered fungal community composition. SOC increased significantly with reduced management intensity and was strongly correlated with decomposition rates and fungal community composition, suggesting a central role of fungal-driven processes in organic matter stabilisation. In contrast, TEA was not linked to management intensity but was primarily governed by abiotic factors, especially soil pH. ESU showed no clear response to management but was associated with bacterial community composition. These divergent patterns highlight that different soil processes are controlled by distinct drivers and cannot be captured by a single process. Our results demonstrate that local-scale management decisions influence soil processes through complex and non-linear pathways shaped by both abiotic and biotic factors. These findings underscore the importance of multi-process approaches and context-sensitive management strategies to support sustainable grassland use.

3.1 Introduction

In Europe, the agricultural system is undergoing a transformation aimed at greater sustainability, following a dual trajectory. On one hand, intensification continues and is supported by technological innovations such as precision agriculture that aim to increase production efficiency and reduce nutrient losses.. On the other hand, there is a growing shift toward less intensive management (i.e. extensification), including organic, regenerative, and nature-inclusive farming systems (Van Grinsven et al., 2015). Extensification seeks to reduce environmental impacts and resource depletion while maintaining food production by reducing external inputs (e.g. chemical fertilizers), increasing aboveground biodiversity, and lowering livestock densities, while enhancing critical soil functions to sustain productivity (Bender et al., 2016; Bommarco et al., 2013; Van Grinsven et al., 2015). A central component of this approach is the improvement of key abiotic soil conditions such as soil organic carbon and pH, and the stimulation of soil life. Together, these abiotic and biotic factors underpin soil functioning (Creamer et al., 2022; Nazaries et al., 2021; Zhou et al., 2020). However, the mechanisms through which agricultural practices influence these factors, and ultimately soil functioning, remain poorly understood under real-world, on-farm conditions (Bardgett & van der Putten, 2014; Nazaries et al., 2021).

Transitioning from intensive to extensive management practices can influence soil abiotic and biotic factors in multiple pathways. For example, shifting from inorganic chemical fertilizer to organic manure can increase complex organic matter content in the soil, potentially resulting in microbial communities that are more adapted to these substrates Francioli et al. (2016) and Hartmann et al. (2015) in arable fields and Lin et al. (2019) in temperate grasslands. Additionally, plant diversity may affect the microbial community through exuding a greater variety of specific exudates and producing contrasting litters, which could lead to distinct microbial communities (Steinauer et al., 2016; Wardle et al., 2004). Furthermore, decreased grazing intensity affects soil organisms through multiple mechanisms, including changes in plant biomass input, substrate availability, soil conditions such as potential shifts in pH and bulk density (Wang et al., 2020). In turn, management-induced changes in the soil microbial community composition can enhance soil functioning, by promoting specific functional groups (Köhl et al., 2014).

Beyond the multiple pathways through which management practices influence abiotic and biotic soil parameters, changes in soil communities often emerge slowly, ranging from 2-10 years (Briar et al., 2007; Schrama et al., 2018; Verbruggen et al., 2010). Shifts in microbial composition and their consequences for soil functioning may only become apparent several years after conversion, due to legacy effects of intensive management (Hartmann & Six, 2023; Schrama et al., 2018; Serrano-Grijalva et al., 2024). Understanding how

these communities and their associated functions evolve over time is therefore essential for farmers who aim at increased sustainable land management. Because of this slow response, fields that have been consistently managed for more than a decade provide an opportunity to examine how different levels of management intensity translate into soil functions in the long term. Studying management intensity gradients - where fields differ in intensity but share similar environmental conditions - offers a possibility to disentangle the effects of management from background variability (Giller et al., 1997), especially when focusing on functions that are sensitive to management and directly linked to agroecological outcomes such as nutrient cycling.

Efficient nutrient cycling is necessary for maintaining crop yields under more extensive management, as farmers increasingly depend on natural soil processes to supply essential nutrients (Köhl et al., 2014). Like other soil functions, nutrient cycling is governed by the interplay of physical (e.g., soil structure), chemical (e.g., soil organic carbon, nitrogen, pH), and biological (e.g., soil micro- and macrofauna, plant roots), factors and processes (Bünemann et al., 2018; Creamer et al., 2022). The depolymerisation, decomposition, and mineralisation of organic matter are critical pathways which drive nutrient availability. These processes are mediated by diverse communities of soil organisms whose activity is shaped by abiotic conditions such as bulk density, pH, SOC, and nitrogen availability, while they also actively modify these properties (Bardgett & van der Putten, 2014; Creamer et al., 2022; van der Heijden et al., 2008; Wagg et al., 2014).

Here, we investigate how variations in current grassland management regimes (based on farmers surveys) along a management gradient influence soil microbial communities and abiotic soil properties, and how apparent changes therein affect multiple soil processes related to field-scale soil functioning. Since nutrient cycling comprises multiple interlinked sub-processes, relying on a single measurement for a soil function can lead to biased interpretations of this function by not fully representing the underlying complexity of soil functioning (Creamer et al., 2022). To accurately capture this complexity, we adopt a multi-process approach using three widely used methods (Fig. 1): 1) enzymatic activity assays (Sinsabaugh et al., 2008), 2) the Teabag Index (Keuskamp et al., 2013), and 3) MicroResp (Campbell et al., 2003).

These methods assess individual processes coupled to organic matter degradation, to allow the evaluation of the microbial community's capacity to: (1) depolymerise organic matter, 2) decompose standardized litter, and (3) mineralize a diverse range of substrates. These methods can be sensitive indicators of changes in management intensity (Bongiorno et al., 2020; Cenini et al., 2016; Hayes et al., 2024), and so together, collectively assess the overall ability of a soil community to degrade organic matter, and to effectively

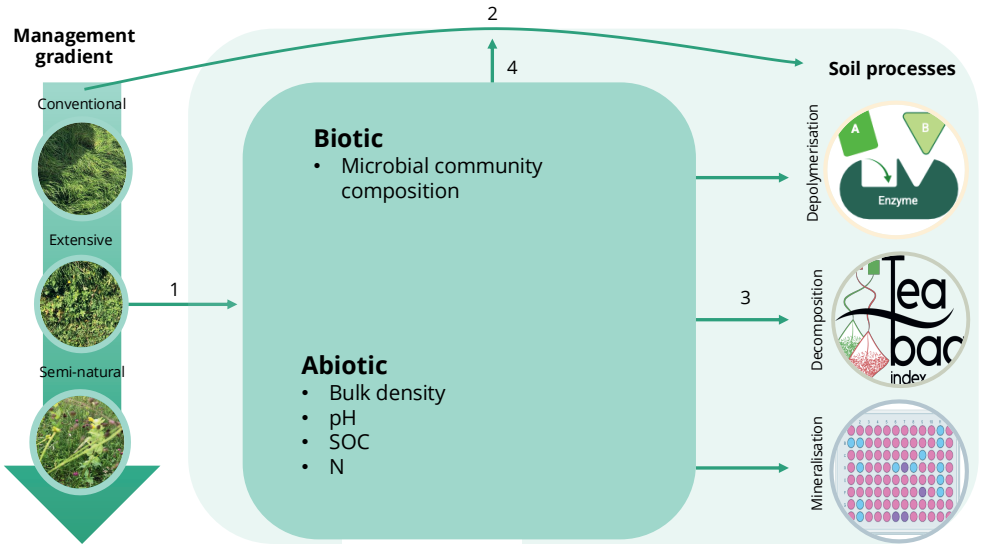


Figure 3.1: Methodological framework of the study, with the numbers indicating the hypothesis listed in the introduction

release nutrients to the soil. Potential effects of climatic and environmental variability were minimised as we conducted our work at a local scale; all fields were situated within a 12 km radius.

We hypothesize (Fig. 3.1) that (1) lower grassland management intensity will lead to higher soil organic carbon (SOC), nitrogen (N), and pH, and decreased bulk density, compared to higher management intensity. These changes are expected to drive shifts in microbial community composition. We also hypothesize that (2) lower management intensity will yield higher rates of soil processes (i.e. depolymerisation, decomposition, and mineralisation). Finally, we expect that (3) variation in soil processes is related to both microbial community composition and abiotic soil properties, and that (4) models integrating both microbial and abiotic predictors will explain more variation in soil functioning than models including only one predictor type. To test these relationships, we use linear mixed-effects models to evaluate interactions among management, soil parameters, and functioning.

3.2 Materials and methods

3.2.1 Grassland selection

We conducted our experimental survey in the Ooijpolder region (Netherlands, 51°51'42.0"N 5°54'17.2"E), where grasslands on river clay soils are subject to varying management intensity. This region provides a diversity of management practices in the context of otherwise similar conditions of soil type, climate, and landscape. Within this area, we selected 18 permanent grasslands (older than 10 years) situated within a 12 km radius. Fields ranged from conventionally managed grasslands (dominated by perennial ryegrass and subject to chemical fertilization), to extensive grasslands (herb-rich swards without chemical fertilizer input), and semi-natural grasslands (herb-rich swards no longer used for production) (n= 6). A detailed overview of the management characteristics for each field is provided in Supplementary Table S3.1.

3.2.2 Soil sampling and analyses

Soil samples were taken in October-November 2022 as described in Boone et al. (2025). Briefly, per field three 2 x 2 m plots were randomly selected. Within each plot, soils were sampled at a depth of 0-25 cm at twelve points (following a W-shape) and combined to form one plot-specific bulk composite sample, resulting in three composite samples per field. Samples were sieved at 2 mm, after which a subsample was stored at -20 ºC for microbial community and biochemical analysis. One part of the remaining sample was stored at 4 ºC and the other dried for subsequent chemical analysis (see Boone et al., 2025 for further details). Briefly, soil pH was measured in a NaCl soil extract from the fresh soil sample. Soil organic matter (%) was determined with loss-on-ignition at 500 °C for 4 hours. Soil organic carbon (%) and nitrogen (%) were determined on 20-30 mg dried and grounded soil samples using an Elemental Analyser (NA1500, Carlo Erba, Thermo Scientific, U.S.A.). Dry bulk density (BD) was assessed using 100 cc soil cores at depths of 0-5 cm and 5-10 cm. Fractions larger than 2 mm were removed from the total weight and volume before analysis. The values were averaged to derive a bulk density value for the 0-10 cm profile. To calculate the mineral bulk density, the resulting bulk density was corrected for organic matter content using the following formula: BD = BDtotal / (1 - (Organic Matter/100)). A detailed description of the sample processing methods and biochemical analysis can be found in the Supplementary Method section.

3.2.3 Soil processes

To assess the soil's capacity to degrade organic matter and release nutrients, three methods were chosen based on their suitability and sensitivity. This multi-process approach enabled a comprehensive understanding of the interconnected sub-processes involved in soil nutrient cycling.

Decomposition rate

The Teabag Index (TBI) was used to measure the effect of management on decomposition and stabilisation of organic matter by the microbial community in the fields. Green Tea and Rooibos tea (Lipton) were installed during the growing season of 2022 (June - September) following the protocol by Keuskamp et al. (2013) with minor adaptations. In each corner of a subplot (hence four per plot), a Rooibos and Green teabag was installed in holes at a depth of 8 cm, with the holes 15 cm apart. Teabags were left for c. 100 days. After recovery, teabags were freeze dried, not airdried as by Keuskamp et al. (2013), to determine dry weight. Teabag Index indicator k (early decomposition rate constant) was calculated following Keuskamp et al. (2013) and used in further analysis indicated as "Decomposition".

Depolymerisation of organic matter

Extracellular enzymes, produced primarily by soil microbes and to some extent by plant roots, catalyse the breakdown and depolymerisation of complex organic compounds (Sinsabaugh et al., 2008). To assess the activity of the soil communities in this depolymerisation process, we performed Extracellular Enzymes (EE) assays to determine the potential of hydrolytic enzymatic activity, following (Jassey et al., 2012). Soil enzymes were extracted by mixing soil with an extraction solution (0.1 M $CaCl_2$, 0.05% Tween 80 and 20 g of polyvinylpolypyrrolidone). Suspensions were centrifuged, filtered and dialysed. Concentrated solutions were split and one part was boiled at 90 °C for 1 hr to denature all enzymes. Samples (boiled and unboiled) were pippeted onto 96-well plates on which the following substrates were added; 4-MUB-α-D-galactoside, 4-MUB-β-D-xyloside, 4-MUB - phosphate, 4-MUB - sulfate, 4-MUB-N-acetyl-β-glucosaminide and L-Leucine 7-amido-4-methylcoumarin. Samples were incubated at 25 °C for 3 h in the dark. The final potential activity was calculated by subtracting the boiled sample values from the corresponding unboiled values, and values were expressed in nmol MUF/MUC released per gram of dry soil per h (nmol g^{-1} h^{-1}). To convert all enzymatic activity into one parameter, each enzyme value was first transformed with log(x+1) and then summed

across all eight enzymatic activity assays into one composite index per sample, referred to as Total potential Enzymatic Activity, TEA (Piton et al., 2020)

Mineralisation

The potential of the microbial community to break down a selection of soil specific substrates, was measured using MicroRespTM, following (Campbell et al., 2003) with minor adaptations. Briefly, colorimetric detection plates were created by mixing cresol red indicator with agar. Eight substrates were selected based on their relevancy for agricultural soils (Bongiorno et al., 2020; Creamer et al., 2009); glucose (GLUC), lignin (LIG), N-acetyl glucosamine (N-ACETYL), gamma amino butyric acid (GAMA), alpha keto-glutaric acid (AKA), oxalix acid (OX), L-alanine (ALA), and miliq as a control. Prior to analysis, soils were incubated for 7 days in the dark at 20 °C, at 30-60% water holding capacity. After incubation, soils were weighed into 96 deep-well plates and substrates were added to the soils at a concentration of 30 mg/g soil water. Plates were then sealed with the colorimetric detection plates and incubated at 20 °C for 6 hours to measure CO_2 respiration. Respiration rates of the substrates (SIR) were calculated as the difference between absorbance values before and after incubation (ug CO_2 g-1 C h-1). To account for basal respiration (e.g. respiration not induced by the addition of substrates), SIR values were divided by the basal respiration. All individual SIR values were summed, resulting in a Multiple Substrate Induced Respiration value (MSIR). SIR values were then converted to relative utilisation by dividing it by MSIR of a sample. With the relative utilisation, the evenness of the substrate utilisation (H') was calculated following (Bongiorno et al., 2020), referred to in this study as Evenness of Substrate Utilisation (ESU) onwards. Technical replicates were averaged per sample and calculations were made accordingly. Although pre-caution was taken to avoid effects of CaCO3 evaporation (see supplementary methods), the strong correlation of AKA and OX with pH may still have been a result of $CaCO_3$ evaporation effects. Hence, these data were removed from all calculations.

3.2.4 Plant and microbial communities

Plant species richness

Per plot (2 m^2), plant species richness was recorded in May-July 2023 using the Braun-Blanquet method (Westhoff & van der Maarel, 1978). For each plot, the total number of distinct plant species identified was used as a measure of plant species richness. To obtain a representative value at the field level, species richness values from individual plots were averaged.

Soil microbial community composition

Soil microbial DNA was extracted according to (Boone et al., 2025). In short, soil microbial DNA was extracted from 0.25 g of sieved soil using the DNeasy PowerSoil Pro kit (Qiagen, China), followed by purification with the gDNA Clean Up kit (Qiagen, China). DNA was sent to Novogene (Cambridge, UK) for metagenomic amplification and sequencing. The 4V region of the 16S rRNA gene was amplified using 515F/806R primers (Caporaso et al., 2012), and the ITS2 region with ITS3-2024F/ITS4-2409R primers (White et al., 1990). PCR was performed using Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Sequencing was conducted on an Illumina NovaSeq 6000 with 250-bp paired-end reads (Novogene, Cambridge, UK). Sequences were processed in Dada2 (version 1.8) (Callahan et al., 2016) for quality filtering, trimming, and taxonomic classification using the SILVA SSU database for 16S (version 138, 2019) (Quast et al., 2012), and for ITS2 using the UNITE fungal database (version 9.0) (Kõljalg et al., 2013; Nilsson et al., 2019). Although 16S rRNA gene sequencing also captured a small number of archaeal sequences (n = 42 out of 9,413 total sequences, <0.5%), communities are referred to throughout as 'bacterial' for simplicity, as the archaeal fraction is negligible and does not materially affect the analyses or interpretations presented. For the ITS2 data, an additional blast was performed manually to remove plant related sequences. Amplicon Sequencing Variant (ASV) tables generated from the 16S and ITS2 sequences were used for further analysis. Further details on the processing of the microbial community data can be found in the Supplementary Method section.

3.2.5 Statistics

Management gradient

All statistical analyses were performed in R, using a significance threshold of $p < 0.05$. Rather than relying on predefined management categories, we aimed to capture the variation in current management practices and their relation to soil function. To characterize field-level management, we conducted farmer surveys, collecting data on fertilization, pesticide use, grazing, mowing, and plant species richness (assessed at the plot level and later averaged to field level). Management information originated from the same farmer survey dataset used in previous analyses (Boone et al., 2025), but the present study focuses specifically on capturing continuous variation in management intensity rather than identifying discrete management clusters. All selected fields had been managed consistently under the same regime for at least the past 10 years. We also recorded the age of each grassland (i.e., time since conversion to grassland) and the duration of the current management regime (i.e. how long the soil has been exposed to the given set of

conditions). Based on these data, we constructed a management gradient that positioned fields along a continuum from intensive to extensive management. Here, we emphasize that this gradient by no means reflects the full legacy of past land use, but highlights current practices. To reduce redundancy among management variables, we assessed collinearity using Spearman's r, applying a threshold of $r > 0.85$. Even though the two major fertilization pathways (chemical N inputs and organic manure inputs) exhibited strong pairwise correlations (>0.85), we retained these measures because they capture distinct ecological dimensions of management intensity and thereby contribute essential structure to the multivariate gradient. The remaining variables (Table S3.2; grassland age, management age, annual grass cutting frequency, months of grazing, livestock units per hectare, chemical nitrogen input per hectare, organic manure input per hectare, and plant richness) were scaled (centering by mean and scaling by standard deviation). The scaled management variables were used to derive a management gradient using Sparse Principal Component Analysis (sPCA) (Rohart et al., 2017) implemented in the mixOmics package (spca function). Sparse PCA identifies a set of sparse principal components that optimally reconstruct the data while forcing some coefficients to zero. We configured the sparsity so that, on average, each management variable appears only once among the sparse principal components, enhancing interpretability relative to standard Principal Component Analysis (PCA). Compared with PCA, Sparse PCA (sPCA) yields a similar total explained variance but produces more interpretable loadings at the cost of perfect orthogonality among components. Variable contributions to each sPC axis were extracted to assess balanced contributions. As management gradient we used the first sPC axis and also visualised the first two sPCs as a two-dimensional gradient. We evaluated the sensitivity of the sPCA by systematically omitting each management variable in turn (leave-one-out approach; Josse & Husson 2012). For each reduced dataset, sPCA was recalculated, and the resulting component scores were compared with those of the full model to quantify the influence of each variable on the ordination and assess the robustness of the management gradient.

Biotic and abiotic soil parameters

Microbial community composition (bacterial and fungal) was analysed using Bray-Curtis distances, based on 16S and ITS2 ASVs within the microeco package. Ordination was performed using Principal Coordinates Analysis (PCoA) on the Bray-Curtis distance matrix. The PCo1 axis for both 16S and ITS2 (explaining 25.0% and 18.8% of the variation, respectively) were extracted to serve as a community composition gradient for further analyses (Fig. S3.1A & S3.1B). The effect of the management gradient on the biotic and abiotic soil pa-

rameters was tested with linear mixed effect models. Statistical inference was based on linear mixed-effect models using plot-level data with Field as a random factor with the lmer function from the lme4 package (Bates et al., 2015). For visualization, all abiotic and biotic parameters were averaged per field to show field-level patterns along the management gradient (Fig. 3.3-3.5) with ggplot2 (Wickham, 2016). When the effect of the management gradient was significant, a regression line was visualised using "lm" for the averaged data. Apart from this, the distribution of individual soil processes was visualized using scatter plots with LOESS regression and 95% confidence intervals (ggplot2).

To explore the multivariate structure underlying all three soil processes, we performed a PCoA using Bray-Curtis distances of normalized metrics (each value divided by the mean of the individual process). To link PCoA patterns to microbial community composition, we extracted the most explanatory PCo axis and correlated it with microbial community gradients using Pearson (for parametric data) and Spearman (for non-parametric data) correlations. Additionally, Procrustes analysis was conducted to assess alignment between ordinations, with significance tested using the protest function from the vegan package (Oksanen et al., 2025). Correlation matrices between variables were generated using Spearman rank correlations. To identify key drivers of soil functioning, we conducted model selection using linear mixed-effect models (lmer function from the lme4 package) for each individual soil process as a function of the ME gradient, biotic, and abiotic variables. Plot-level data were used, with "Field" included as a random effect to account for the nested experimental design. The dredge function identified the best-fitting model based on the lowest AIC value. Variance inflation factors (VIF) were calculated to check for multicollinearity, and variables with VIF > 5 were excluded. This approach allowed us to evaluate the relative importance of biotic and abiotic factors in explaining soil functioning while accounting for ME and plot-level variation. The significance of fixed effects was determined using ANOVA, using the anova function within the lme4 package.

3.3 Results

3.3.1 Management gradient

The management gradient did not show strong clustering of fields (Fig. 3.2). The Leave-one-variable-out analysis showed that the distribution of the fields within the sPCA was robust (Table S3.3). Across all eight management variables, correlations between the full and reduced models exceeded 0.99 for sPC1 and 0.96 for sPC2 (except for sPC2 when removing grassland age). Mean shifts in field positions were small (<1), indicating that no single management variable overly influenced the distribution of fields in the sPCA. The first sPC

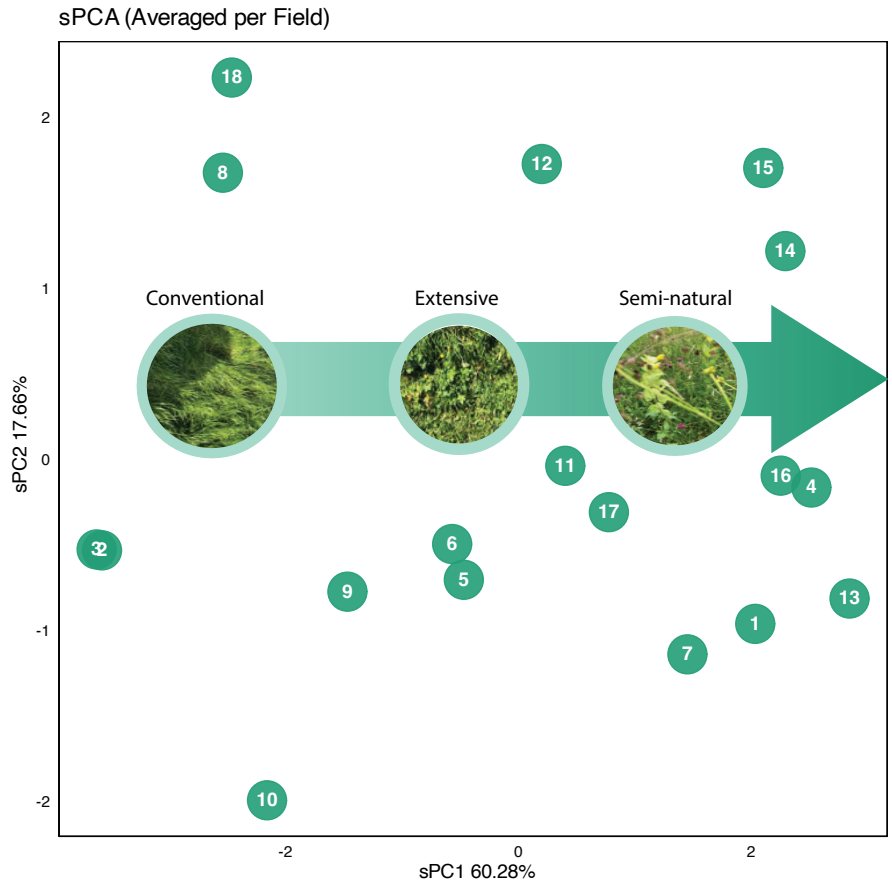


Figure 3.2: Ordination plot of the first two sparse PC axes (sPC1 explaining 58.69% of the variance; sPC2 explaining 17.00%) showing the position of each field (averaged per field) based on farmer-surveyed management variables. Numbers denote field IDs; proximity of points reflects similarity in management profiles.

axis - explaining 60.28% of the variation - was then used to signify a management gradient. Along this sPC1, fields ranged from most intensive practices (conventional grasslands, -3.6) to least extensive practices (semi-natural grasslands, 2.8). Organic manure input (m^3 ha-1), plant species richness, and chemical nitrogen input (kg N ha-1) contributed most to the distribution (16.30%, 15.22%, and 15.15%, respectively). The gradient represents the relative position of the fields based on the management practices applied over the past 10 years. "Age of Grassland" was the only variable that was highly related to sPC2 (38.22%, Table S3.2).

3.3.2 Management gradient and microbial community composition

Analysis of the microbial community showed no significant effect of the management gradient on the composition of the bacterial community ($F_{1,17}$ = 2.02, p =0.17; Fig. 3.3A). The composition of the fungal community, however, was strongly correlated with the management gradient ($F_{1,17}$ = 36.20, p > 0.001, R^2m = 0.62; Fig. 3.3B). Among the management variables used to define the management gradient, most variables, except for "Age of Grassland" and "Age of Management", were correlated strongly with the shift in fungal community composition (Fig. S3.2). All variables were negatively correlated with fungal composition, except for "Plant Richness," which showed a strong positive correlation (r = 0.81).

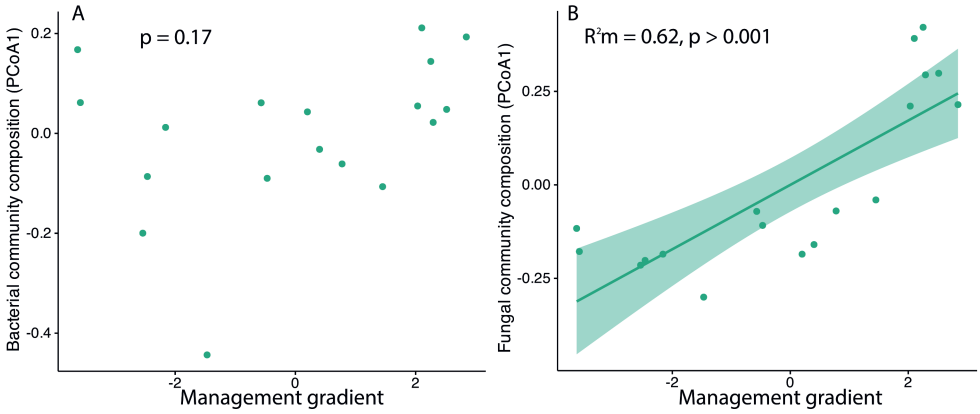


Figure 3.3: Relationships between the management gradient and A) bacterial and B) fungal community composition gradient. Shown are their linear regressions of the first PCoA axis (PCOA1) scores for A and B, plotted against the management gradient, with their respective Spearman and Pearson's correlation coefficients (R) and p-values. For B), shaded areas represent 95% confidence intervals.

3.3.3 Management gradient and soil abiotic variables

Soil physical and chemical properties showed varying responses across the management gradient, with several patterns contradicting our hypotheses (Fig. 3.4). Bulk density was not affected by the management gradient, with values ranging between 0.9 and 1.3 g cm-3 across the gradient ($F_{1,17}$ = 0.89, p =0.36; Fig. 3.4A). Soil pH showed a bimodal distribution with two distinct clusters (pH 5.5-6.0 vs. 7.0-7.5), likely reflecting historical management practices (liming), as no significant correlation was found between pH and the management gradient ($F_{1,25}$ = 0.77, p =0.39; Fig. 3.4B).

Soil organic carbon (SOC) was positively related to the management gradient ($F_{1,17}$ = 5.90, p =0.03, R^2m = 0.22; Fig. 3.4C). While one outlier (6%) could inflate the model, when excluding the outlier from the regression analysis, the significant effect of the management

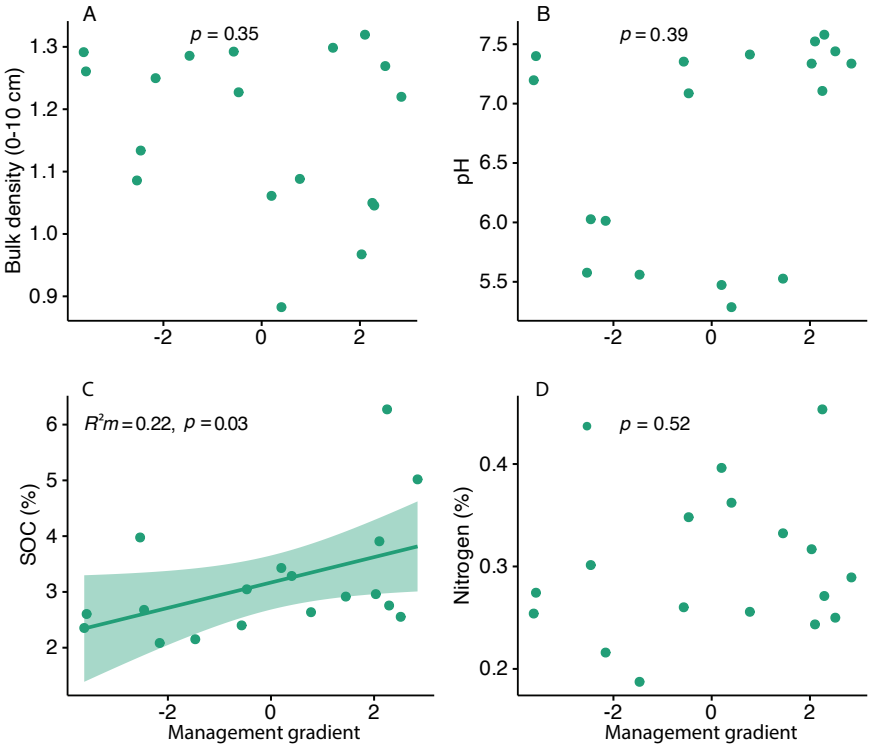


Figure 3.4: Variation in soil abiotic properties along the management gradient. Shown are A) Soil dry bulk density (0-10 cm), B) soil pH, C) Soil Organic Carbon (%) and D) Total Nitrogen (%). Data points represent individual plots along the management gradient, with fitted regression lines and 95% confidence intervals where applicable. Corresponding correlation coefficients (R) in case of linear relation and p-values are indicated.

gradient on SOC was still present ($F_{1,18}$ = 4.71, p =0.04). Each management practice showed a strong correlation specifically with SOC (%) (Unit of Livestock, Amount of Chemical Manure, and Amount of Organic Manure; Fig. S3.2). Soil nitrogen content was not related to the management gradient ($F_{1,17}$, p =0.52; Fig. 3.4D), with values ranging from 0.2% to approximately 0.45% across the gradient, contrary to our hypothesis.

3.3.4 Management gradient and soil processes

The three soil processes representing different aspects of OM decomposition - enzymatic depolymerization (TEA), standardized litter decomposition (k), and substrate mineralization (ESU)—showed contrasting response patterns to management along the gradient (Fig. 3.5, Fig. S3.3 & Fig. S3.4). Total enzymatic activity (TEA) displayed considerable variability along the gradient and displayed a weak mid-gradient rise, followed by a decline at the extensive end; however, neither linear or non-linear models showed to be statistically significant ($F_{1,16}$ = 0.132, p = 0.72; Fig. 3.5A), contradicting our hypothesis of a simple positive relationship with extensification. In contrast, decomposition rate constant k did show a significant positive correlation with the management gradient ($F_{1,16}$ = 5.18, p = 0.37; Fig 3.5B). Evenness of substrate utilization (ESU) exhibited a no discernible pattern across the management gradient ($F_{1,16}$ = 0.173, p = 0.68; Fig. 3.5C).

Shifts in soil processes along the management gradient also appeared to co-vary with shifts in community composition and abiotic parameters. For the individual soil processes, the bacterial community gradient showed a strong correlation with ESU (Pearson r =0.77, p = > 0.001; Fig. 3.6), while the fungal community showed significant correlations with both decomposition (Pearson r =0.60, p =0.01) and TEA (Pearson r =0.48, p =0.04), but not with ESU. In addition to testing correlations with individual soil processes, we examined whether shifts in overall soil functioning were associated with changes in microbial community composition. Functional shifts, as represented by the first two axes of the soil process PCoA, were moderately aligned with microbial community composition (as assessed by Procrustes analysis). For bacterial communities, we found a borderline significant but moderate association (cor =0.46, m^2 = 0.78, p =0.055), while fungal communities showed a slightly stronger significant association (co$r$ =0.56, m^2 = 0.67, p =0.004). As for correlations between soil abiotic factors and individual soil processes distinct patterns emerged (Fig. 3.6). While both bulk density and pH exerted a strong negative correlation with TEA (Pearson r =-0.50 and r =-0.76, respectively), N showed a positive association (Pearson r =0.48). Furthermore, both N and SOC showed a strong positive correlation with Decomposition (Pearson r =0.50 and r =0.56, respectively). ESU was the only soil process that did not show a significant correlation with any of the abiotic variables.

Linear mixed-effects models revealed that different soil processes were governed by distinct combinations of biotic and abiotic factors, providing mixed support for our hypothesis that combined models would be superior (Table 3.1). For TEA, the best model included nitrogen (%), pH, and bulk density as predictors, with pH showing the strongest effect (p =0.0002) while the management gradient had no significant effect (p =07.79). The model explained 46.8% of the variation for fixed effects and 48.6% including “Field” as ran-

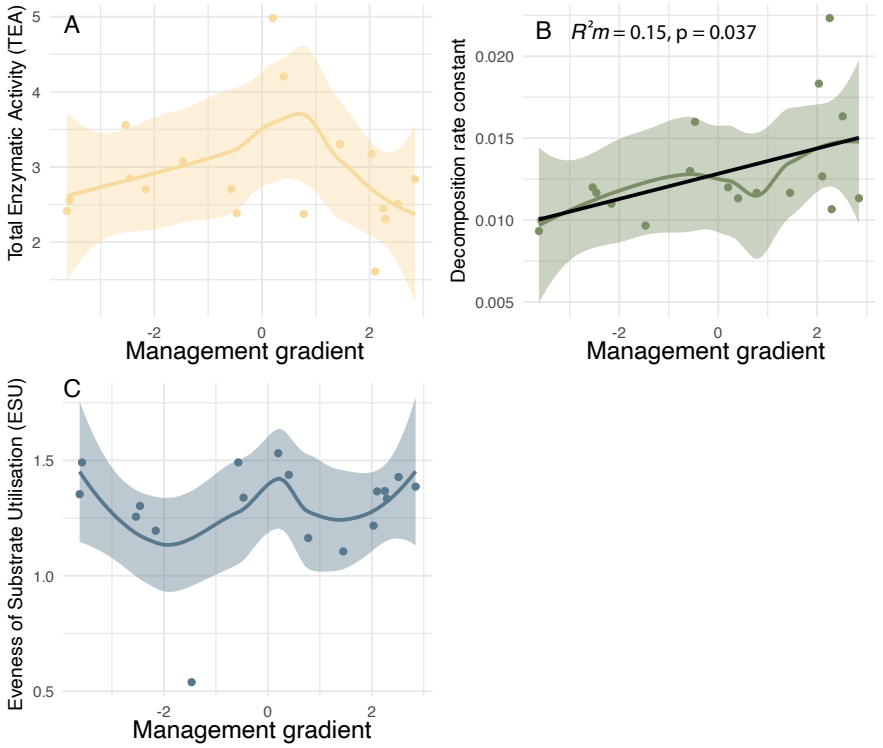


Figure 3.5: Responses of soil processes along the management gradient; A) Total Enzymatic activity (sum of all log(1+x) transformed enzyme activities (MUF/MUC nml/h/g), B) Decomposition rate constant (k, Teabag index), C) Evenness of substrate utilisation (ESU). LOESS regression curves with 95% confidence intervals are shown to illustrate the non-linear trends across the gradient. While TEA and ESU exhibit variable non-significant responses, Decomposition rate shows a significant positive linear trend, illustrated in B, with its corresponding Pearson's correlation coefficient R and p-value.

dom effect, indicating that Field did not contribute to the overall explanatory power. The bimodal pH distribution had important functional consequences. TEA was significantly higher in more acidic soils (pH < 6.2) compared to alkaline soils (pH > 7.0) (F = 13.59, p < 0.05). However, the management gradient effect on TEA remained non-significant within both pH groups (acidic soils: F = 2.46, p =0.18; alkaline soils: F = 0.001, p =0.97), indicating that pH affects enzymatic activity independently of management intensity. For decomposition, model performance improved when incorporating fungal community composition, although this yielded only a marginal effect (ANOVA; p =0.08). The final model explained 22.4% of variation for fixed effects, and 47.7% if field effects are included. For ESU, the model fit improved with the inclusion of the bacterial community composition as a predictor, again indicating that this process is solely associated with the bacterial community composition. Here, bacterial community composition showed to have a significant effect

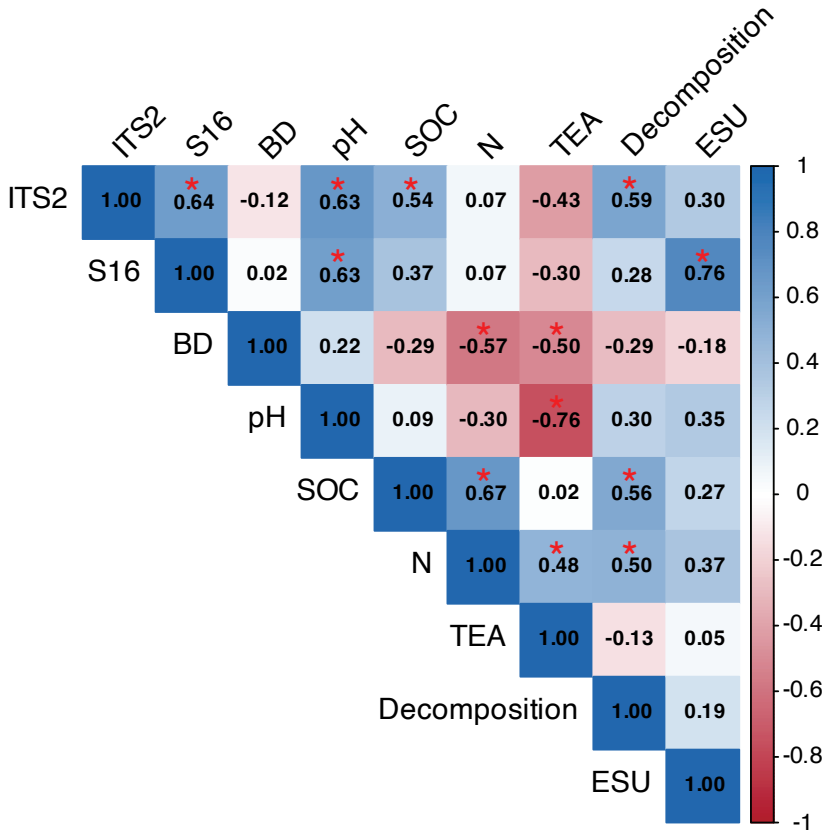


Figure 3.6: Pearson correlation matrix showing pairwise correlations among soil variables. Asterisks indicate statistically significant correlations ($p < 0.05$). BD = bulk density; SOC = soil organic carbon (%); N = nitrogen (%); TEA = total enzymatic activity; Decomposition = decomposition rate (k, teabag index); ESU = evenness of substrate utilisation (MicroResp).

on ESU (ANOVA; p =0.001). However, the fixed effects of the final model could only explain 18.7% of the variation, and the inclusion of field as random effect did not increase this share.

3.4 Discussion

Our study shows that spatial variation in grassland management intensity is reflected in several, but not all, properties and processes of the soil system. Some patterns emerged clearly: the management gradient was strongly associated with fungal community composition, SOC and litter decomposition rates, indicating that management influences both biotic and abiotic processes. Other soil properties and processes however, (including bac-

Table 3.1: Mixed-effects model results for enzyme activity, decomposition, and respiration along the management intensity gradient. Asterisks (*) indicate significance at $p < 0.05$.

Model	Variance explained by Fixed effects / Field & Fixed effects	Fixed effect	Estimate	p-value
Enzymes ~ management gradient + (1 | Field) + N + pH + BD	46.8% / 48.6%	Management gradient	0.01	0.79
		Nitrogen (%)	1.80	0.21
		pH	-0.58	0.0002*
		Bulk density	-1.35	0.10
Decomposition ~ management gradient + (1 | Field) + ITS2	22.4% / 47.7%	Management gradient	0.0001	0.79
		ITS2 comm. comp.	0.01	0.08
Respiration ~ management gradient + (1 | Field) + 16S	18.7% / 18.7%	Management gradient	-0.01	0.65
		16S	0.87	0.00113*

terial community composition, bulk density, pH, nitrogen, TEA and ESU) showed weak or inconsistent relationships with management. Together, these findings suggest that a change in management does not drive the soil system in a uniform direction. Instead, management affects a suite of soil processes in divergent ways, with some responding predictably to management practices and others being shaped more strongly by local abiotic constraints.

3.4.1 Validation of the management gradient

This study was based on a gradient of current management practices, ranging from most to least intensive. We recognize, however, that soil responses can show hysteresis: changes in soil properties and functions may lag behind, or follow alternative trajectories, depending on historical land use (Liiri et al., 2012; McLauchlan, 2006). The absence of detailed records on pre-conversion management further limits our ability to fully separate legacy effects from recent practices. As stated by the European Union, "extensification of farming is the opposite of intensification" (ec.europa.eu). Yet in practice, due to soil hysteresis and land-use legacies, extensification does not necessarily follow the reverse trajectory of intensification. For example, historical pesticide usage can have long-lasting impacts on the soil organisms (van Eekeren et al., 2022). Although we did not assess pesticide residues in this study, such findings illustrate how past management can continue to influence current soil life.

While our gradient can be confidently described as an intensification gradient, it should not automatically be interpreted as a true extensification gradient. For this reason, our analyses focused on associations between current management and soil functioning, without assuming full ecological reversibility of prior impacts. Importantly, all fields have been managed consistently for at least a decade, which supports the assumption that we are observing long-term responses; soil process changes are known to emerge within 2-10 years (Briar et al., 2007; Schrama et al., 2018; Verbruggen et al., 2010). Furthermore, the 'Age of Grassland' in our study was > 20 years and 'Age of Management' was > 10 years, and contributed mainly to PC2 (Table S3.2), indicating that PC1—the axis used to construct our management gradient, primarily captured variation independent of historical factors.

3.4.2 Differential responses of bacterial and fungal communities

Partly contrasting our hypothesis, despite their communities being strongly correlated (Spearman R = 0.64, Fig. 3.5), bacterial and fungal communities responded differently to an increase in extensive management practices (management gradient). Fungal communities were the most responsive to the management gradient and their association with shifts in overall soil functioning were stronger (Procrustes cor = 0.56, $m\check{s}$ = 0.67, p = 0.004). The fact that fungal communities responded stronger to management is in line with a recent study that showed that fungi and plant communities showed the strongest homogenisation under intensive management, whereas bacterial homogeneity depended largely on pH variation (Mayerhofer et al., 2025). Specifically, from all management practices, plant species richness and organic fertiliser application showed the strongest associations with fungal community structure, whereas none showed consistent associations with bacterial communities. This pattern is consistent with long-term grassland fertilization experiments demonstrating that plant and fungal communities respond similarly to nutrient inputs and mowing, while bacterial communities exhibit distinct and often weaker responses (Cassman et al., 2016; Leff et al., 2015).

Previous work based on the same sequencing dataset examined a different research question, namely the metabolic activity of the microbial community and its volatile organic compound (mVOC) profile (Boone et al., 2025). Although that study did not assess soil functioning or management-function relationships, the insights gained from its analysis of the microbial community structure remain relevant here. Specifically, along the same management gradient, several fungal endophytes increased toward the extensively managed end of the spectrum, including Tetracladium spp., Pyrenochaetopsis, and Cladosporium(Lazar et al., 2022; Wemheuer et al., 2019). Endophytic fungi are known to play important roles in plant health by providing resistance to stresses such as nutrient deficiency,

and have been highlighted for their importance in sustainable agriculture (Lugtenberg et al., 2016).

Although fungi are often emphasised for their role in degrading recalcitrant organic matter (Hoppe et al., 2016; Mathieu et al., 2013), their association in this study with labile litter decomposition (k) aligns with well-established evidence that fungi facilitate the early stages of litter breakdown. In particular, fungal activity can increase substrate accessibility for bacteria and thereby accelerate initial decomposition (de Boer et al., 2005). Our findings therefore confirm this recognised facilitative role of fungi during the onset of litter decay. However, although fungal community composition showed a negative correlation with TEA, the lack of explanatory power when fungal composition was included in the TEA model, suggests that the influence of fungal communities on enzyme activity may be less direct. This is possibly because abiotic factors, such as pH, had a stronger effect on TEA, possibly overruling the effect of fungal communities on TEA, as reported in Kivlin & Treseder (2014). Another possibility is that fungal communities are more strongly linked to oxidative enzyme activity (not measured in this study), which is closely related to pH, whereas our measurements focused on hydrolase activity, which primarily reflects substrate availability and microbial nutrient demand (Sinsabaugh et al., 2008). In contrast to fungal communities, bacterial communities were not significantly affected by the management gradient. However, they did show a moderate correlation (but less strong) with shifts in overall soil functioning (Procrustes cor = 0.46, $m\check{s}$ = 0.78, p = 0.055), similar to fungi. This was also evident when considering their role in driving ESU. ESU, which measures the evenness of substrate use across a range of carbon sources, was highly correlated with bacterial community composition (R = 0.77), suggesting a strong link between bacterial functional diversity and soil metabolic potential. This aligns with findings by (Tong et al., 2021) who reported that bacterial communities predominantly govern substrate utilization dynamics.

3.4.3 Divergent patterns in soil processes

Among all measured abiotic factors, SOC showed the strongest response to the management gradient, increasing significantly with more extensive management practices. While permanent grasslands are generally known to store more SOC than temporary grasslands or arable fields (van Eekeren et al., 2008), and grassland age has been positively associated with SOC accumulation (Iepema et al., 2022), findings within permanent grasslands under varying management regimes remain inconsistent (Abdalla et al., 2018; Leifeld et al., 2009; Mestdagh et al., 2006; Poeplau, 2021). The effect of management on SOC is often depicted as context-dependent, varying with factors such as soil texture, carbon inputs, and

climate (Hassink, 1997; Lal, 2004; Smith, 2014). Our findings support the view that, at the field scale within a single region, lower-intensity management tends to favour SOC accumulation, likely reflecting the combined effects of multiple management components.

Interestingly, SOC showed significant negative correlations with both chemical and organic manure inputs and Unit of Livestock (Fig. S3.2). As the highest organic matter input in grasslands (i.e. the pool for SOC) comes from belowground (roots) and aboveground plant residues (De Vries et al., 2006), the negative correlations could indicate the impact of these management practices on the amount of plant residues redirected back to the soil. Specifically, grazing has shown to both significantly decrease or increase SOC stocks (Abdalla et al., 2018; Eze et al., 2018). However, as our study looked at management as a combination of practices, it is therefore likely that reduced SOC in more intensively managed fields reflects the cumulative impact of lower root biomass and less aboveground plant residues rather than any single management input alone. Its strong correlation with decomposition and fungal community composition suggests that fungal-driven processes may play an important role in SOC dynamics, although cause and effect cannot be teased apart. This potential role of fungi in organic matter stabilisation is further supported by Morriën et al. (2017), who showed that shifts in fungal community composition and activity (along a chronosequence of abandoned arable fields developing into semi-natural grasslands) led to more efficiently stabilised decomposition products. Additionally, the importance of SOC as a central indicator for soil multifunctionality, as highlighted in arable systems by van Rijssel et al. (2025), may also apply here: our findings suggest that SOC acts as integrative indicator linking abiotic and biotic properties to management intensity. Unlike decomposition and SOC, which increased linearly along the management gradient and were closely linked to microbial community composition, total extracellular enzymatic activity (TEA) did not show a uniform trend. Instead, TEA was best explained by abiotic factors -particularly soil pH, bulk density (BD), and nitrogen content - highlighting that different soil functions are governed by distinct environmental drivers. Soil pH emerged as a significant predictor of TEA, independent of land-use intensity or microbial community shifts. This is consistent with previous findings showing that enzymatic activity is often more sensitive to changes in soil chemistry than to microbial composition (Kivlin & Treseder, 2014; Wang et al., 2021). Mechanistically, soil pH can directly influence enzyme activity by altering enzyme structure and function, with different isoforms operating optimally at different pH levels (Frankenberger & Johanson, 1982). While our enzyme assays were conducted at a standardized pH of 5.6 (near the enzymatic optimum) the wide range of field soil pH values (5.5-7.5) raises the possibility that assay conditions may have partially influenced observed activity patterns. However, if this were the primary driver,

one would expect consistent effects within the respective pH classes, which was not observed.

The independence of TEA from the management gradient, despite its strong negative correlation with pH, suggests two possible mechanisms: a direct pH effects on enzyme kinetics, where lower pH enhances enzyme-substrate interactions, or indirect effects through selection of microbial taxa with different enzyme production strategies. Our data support the latter, as fungal communities shifted with pH but showed opposing relationships with TEA, indicating that pH-driven community turnover may select for taxa with reduced enzymatic investment. These findings align with prior studies showing that soil pH can drive microbial community turnover, potentially altering community-level functional traits (Rousk et al., 2010; Sinsabaugh et al., 2008). Since pH is a manageable soil property, these results suggest that farmers may be able to enhance enzymatic activity both directly (by optimizing abiotic soil conditions) and indirectly, through their influence on microbial community composition. This dual pathway could support efficient nutrient cycling while also promoting the build-up of stable organic matter, as both decomposition and mineralization were marginally positively associated with pH (Fig. 3.6).

In addition, ESU did not respond clearly to the management gradient. However, this process still showed to be significantly affected by shifts of the bacterial community along the gradient. The lack of response was surprising, seen that multiple studies did successfully use MicroResp to show how microbial functioning was influenced by differences in land uses (Bongiorno et al., 2020; Creamer et al., 2009; Moscatelli et al., 2018). It could be that the differences between the land use gradient in this local-scale study were too small for the method to detect. Additionally, bacterial community composition showed to be the driving force behind ESU in our study. As the bacterial community composition was not strongly affected by the management gradient, this could therefore not differ between the fields in our study.

3.4.4 Implications for sustainable grassland management

This study provides a local perspective on the ecological mechanisms underpinning soil functioning in grasslands. Our findings show that local responses can diverge due to context-specific interactions between management, soil properties, and microbial communities. In this hypothesis of a dynamic nexus, soil processes do not follow a uniform trajectory, but rather move through different pathways, reflecting varied underlying drivers and interactions. For instance, Decomposition increased with the management gradient, Total Enzymatic Activity (TEA) followed a unimodal pattern, and Evenness of Substrate Utilisation (ESU) showed little response. These divergent responses emphasize

that no single process or proxy can fully represent the complexity of organic matter breakdown as they are potentially shaped by different combinations of ecological drivers. This highlights the importance of careful proxy selection when assessing soil "multifunctionality". While single indicators may provide partial insights, they risk oversimplifying complex systems and obscuring key interactions. A multi-proxy approach that integrates multiple dimensions of a function may therefore offer a more robust understanding of ecosystem functioning.

By revealing how local management decisions influence diverse soil processes, our work supports the development of more targeted and context-sensitive strategies for sustainable agricultural management. Rather than reducing complexity to binary classifications, embracing continuous gradients in this study has helped reveal important patterns. Still, the associations observed in this study generate hypotheses rather than definitive relations. Testing these hypotheses will require long-term studies that control for initial conditions and disentangle the influence of time and legacy effects. Finally, sustainable grassland management cannot rely on ecological experiments alone. While farmers may be motivated to adopt more ecologically beneficial practices, such transitions also require adequate economic and social support (Witvliet et al., 2024). Moreover, understanding current ecological conditions also necessitates attention to the historical social fabric that has shaped land use over time (Baumgarten et al., 2025). As such, there is a growing need for interdisciplinary research that can help better support farmers in these transitions by integrating social, economic, and historical dimensions into ecological analyses.

3.5 Conclusion

This study demonstrates that long-term variation in grassland management intensity is reflected in soil functioning through multiple, partly decoupled pathways. Rather than driving uniform responses across the soil system, management influenced specific abiotic properties, microbial communities, and soil processes in divergent ways. In particular, fungal community composition and soil organic carbon emerged as strongly associated with litter decomposition, while other processes such as enzymatic activity and substrate utilization were structured primarily by abiotic constraints or bacterial community composition. These findings highlight that different components of nutrient cycling are governed by distinct ecological drivers, even within a relatively small geographic region. Our results underscore the importance of distinguishing between soil processes when evaluating management effects on soil functioning. Proxies commonly used to represent organic matter degradation responded differently to management intensity and were linked to dif-

ferent biotic and abiotic controls, indicating that no single metric adequately captures the complexity of soil functioning. By applying a multi-process approach, we show that apparent inconsistencies among indicators do not reflect noise, but rather reveal the multifaceted nature of soil ecological responses to management.

The strong association between fungal communities, SOC, and decomposition suggests that fungi play a key role in mediating management effects on carbon turnover in permanent grasslands. However, the absence of consistent responses in bacterial community composition and several soil properties emphasizes the influence of local abiotic conditions and historical legacies in shaping soil systems. These context-dependent effects caution against assuming linear or reversible trajectories following management change and highlight the need to consider time lags and hysteresis in assessments of soil recovery.

Overall, our findings suggest that sustainable grassland management cannot be evaluated through single indicators or binary classifications of management intensity. Instead, understanding how management influences soil functioning requires an integrative perspective that accounts for multiple processes, their distinct drivers, and their interactions over time. Long-term, gradient-based studies such as this one provide valuable insights into these dynamics under real-world conditions and can inform the development of management strategies that balance productivity with the maintenance of key soil functions.

3.6 Acknowledgements

We thank the farmers for granting access to their fields and for allowing the use of information on their management practices in our analyses. We also thank the students Taiko van Engen, Jelle Kusters, Anouk Wakely, and Jan-Willem Takke for their assistance with this research. Finally, we acknowledge the analysts Sebastiaan Krosse and Paul van der Ven of the GI at Radboud University for their support with the chemical analyses.

3.7 Data availability statement

Data available from the Zenodo data repository: https://doi.org/10.5281/zenodo.20539936

Chapter appendix

3.A Supplementary information

3.A.1 Soil processes

Decomposition rate

The Teabag Index (TBI) was used to measure the effect of the management gradient on decomposition and stabilisation of organic matter in grasslands. Teabags were installed during the growing season of 2022 (June - September). Teabags were buried in the soil following the protocol of Keuskamp et al. (2013) with minor adaptations, using Green Tea and Rooibos Tea from Pickwick (EAN numbers). To avoid insufficient replication due to lost or damaged teabags upon recovery, per corner in each plot, two bags of each tea type were buried, resulting in 16 teabags per plot. Per location, GPS coordinates were collected with a dGPS (TOPCON). Teabags were left for approx. 100 days. After recovery, teabags were stored at -20 C until further analysis. After inspection of damage or leaking and sorting out the replicates, one of the replicates was selected and further processed. The tea was removed from the bag and cleaned from visible soil particles and roots and transferred into tubes for freeze drying. After samples were dried for at least 24 hours, the tea was weighed again for dry weight. Teabag Index indicators S (stabilisation factor) and k (early decomposition rate constant) were calculated following Keuskamp et al. (2013).

Depolymerisation of organic matter

To further assess the activity of the decomposing community we performed Extracellular Enzymes (EE) assays to determine the potential of hydrolytic enzymatic activity, following Jassey et al. (2012). Briefly, 5 g of soil (previously stored at -20 C) was added to 50 mL extraction solution, consisting of 0.1 M $CaCl_2$, 0.05% Tween 80 and 20 g of polyvinylpolypyrrolidone. Extractions were shaken at room temperature at 150 rpm for 90 min. After, the mixture was centrifuged at 10,000 rpm for 5 min at 4 °C and filtered with Whatman GF/C of 1.2 µm. The filtrate was concentrated overnight using cellulose dialysis tubes of 10-12 kDa and polyethylene glycol (PEG). Concentrated solution was recollected by adding 10 mL of phosphate buffer (pH 5.6) to the dialysis tubes. The concentrated solution

was split into two, of which one part was boiled at 90 °C for 3 hrs to denature all enzymes (boiled extract), and the other was stored at 4 °C until further analysis (unboiled extract). On opaque 96-well micro-plates, first, four technical replicates of 38 µl enzymatic extract of both the boiled and unboiled samples were added, after which 250 µl of enzyme substrate was added. The following enzyme substrates were used; 4-MUB-β-D-cellobioside, 4-MUB-β-D-xyloside, 4-MUB-β-D-glucoside, 4-MUB - phosphate, 4-MUB - sulfate, L-Leucine 7-amido-4-methylcoumarin, 4-MUB-N-acetyl-β-glucosaminide, 4-MUB-α-D-galactoside and Fluorescein Diacetate. Samples were incubated at 25 °C for 3 h in the dark. After, samples were immediately measured for fluorescence intensity with a photo spectrometer at 365 nm excitation wavelength and 450 nm emission wavelength. The final potential activity was calculated by subtracting the boiled sample values from the corresponding unboiled values, and values were expressed in nmol MUF/MUC released per gram of dry soil per h (nmol g^{-1} h^{-1})

Mineralisation

The potential of the microbial community to break down a selection of soil specific substrates, was measured with MicroRespTM within two weeks after sampling. Methods were based on Campbell et al. (2003) with minor adaptions. Briefly, coulometric detection plates were created by mixing cresol red indicator with agar. Eight substrates were selected based on their relevancy for agricultural soils (Bongiorno et al., Creamer et al.). These included Glucose (GLUC), Lignin (LIG), N-acetyl glucosamine (N-ACETYL), gamma amino butyric acid (GAMA), alpha keto-glutaric acid (AKA), oxalix acid (OX), L-alanine (ALA) and miliq as a control. Soils (30-60% WHC) were pre-weighed into 96-deepwell plates (six technical replicates per soil) and were incubated for 7 days in the dark at 20 C. After incubation, the eight substrates were dispensed onto the soils in random order to avoid edge effects. Concentration of the substrates was 30 mg/g soil water. After adding the substrates, plates were left open for 30 minutes to allow for any possible release of carbonates due to the acidity of the substrates. Before sealing of the plates, coulometric values of the detection plates were measured at 570 nm. After sealing, plates were incubated in the dark at 20 °C for 6 hours. Following the incubation, coulometric plates were read again at 570 to measure the final absorbance values. Respiration rates of the substrates (SIR) were calculated as the difference between absorbance values before and after incubation (ug CO2/g C/h). To account for basal respiration (e.g. respiration not induced by the addition of substrates), SIR values were divided by the basal respiration. Technical replicates were averaged per sample and calculations were made accordingly. Although pre-caution was taken to avoid effects of $CaCO_3$ evaporation (see supplementary

methods), the strong correlation of AKA and OX with pH may still have been a result of CaCO3 evaporation effects. Hence, these data were removed from all calculations.

3.A.2 Soil microbial community composition

Soil microbial DNA was extracted according to Boone et al. (2025). From the sieved soil, 0.25 g was used for extraction with Dneasy PowerSoil Pro isolation kit (Qiagen, China) and further purified with gDNA Clean Up kit (Qiagen), which was then eluted in 50 µL of elution buffer. The DNA was shipped on dry ice to Novogene (Cambridge, UK) for metagenomic amplification and sequencing. The 4V region of the 16S rRNA gene was amplified using primers 515F/806R (Caporaso et al., 2012) and the ITS2 region of the ITS rRNA gene with primers ITS3-2024F/ITS4-2409R (White et al., 1990). PCR was performed with 15 µL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 2 µM forward primers, and approximately 10 ng of template DNA. The PCR protocol included an initial denaturation at 98°C for 1 min, 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s, followed by a final elongation at 72°C for 5 min. PCR products were visualized on 2% agarose gel, mixed in equidensity ratios, and purified using the Universal DNA Purification Kit (TianGen, China). Microbial amplicons were sequenced using the Illumina NovaSeq 6000 with 250-bp paired-end reads (Novogene, Cambridge, UK). Barcodes and primers were removed by Novogene before further processing in Dada2 (version 1.8) (Callahan et al., 2016). In Dada2, sequences for both 16S and ITS gene amplicons were quality filtered and trimmed, allowing no ambiguous bases. The 16S truncation length was set at 218 bp for both forward and reverse reads, while ITS2 had a minimum length of 50 bp. To address binned quality scores in NovaSeq data, a modified error rate estimation function was implemented, using local regression ('loess') to model error probabilities based on sequencing positions, with a maximum error estimate of 0.25. Chimeras were removed using the consensus method. Taxonomic classification for 16S was performed using the SILVA SSU database (version 138, 2019) (Quast et al., 2012), and for ITS2 using the UNITE fungal database (version 9.0) (Kõljalg et al., 2013; Nilson et al., 2019). For the ITS2 data, an additional blast was performed manually to remove plant related sequences. Amplicon Sequencing Variant (ASV) tables generated from this process were used for further analysis. For further statistical analyses, bacterial and fungal ASVs were analysed within the Microeco package (Liu et al., 2021). To account for differences in sequencing depth between samples, we used the rarefy function (sample size = 10000). For 16S, 16226 taxa were removed from the ASV table as the abundance was 0 after random subsampling. For ITS2, 7551 taxa were removed.

3.B Supplementary figures

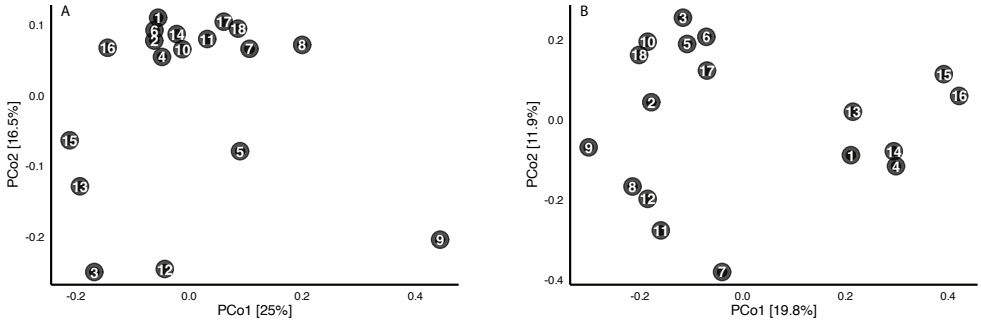


Figure S3.1: First and second Principle Coordinate axis of Principal Coordinate Analysis (PCoA) of A) Bacterial (16S) and B) fungal (ITS2) ASV's, using Bray-Curtis distance matrices.

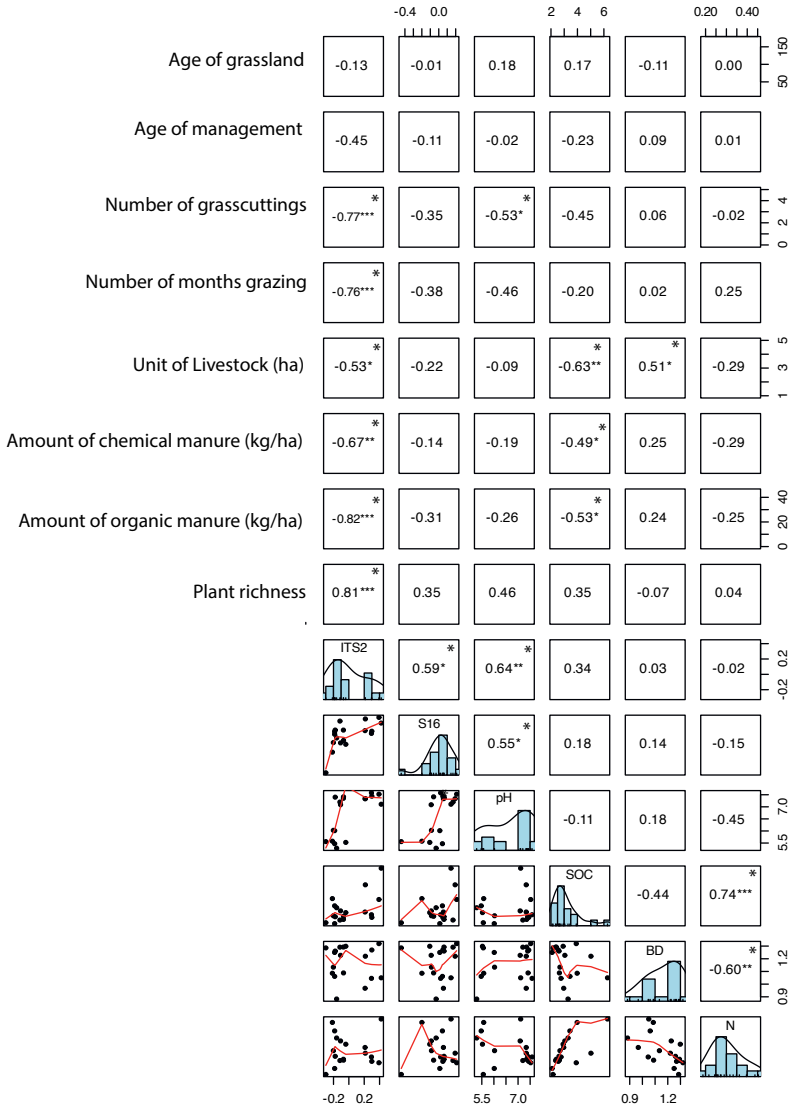


Figure S3.2: Correlation matrix of spearman correlations between individual management variables and biotic and abiotic soil parameters, with significance levels indicated by asterisks : p <0.05 (*), p <0.01 (**), p<0.001 (***).

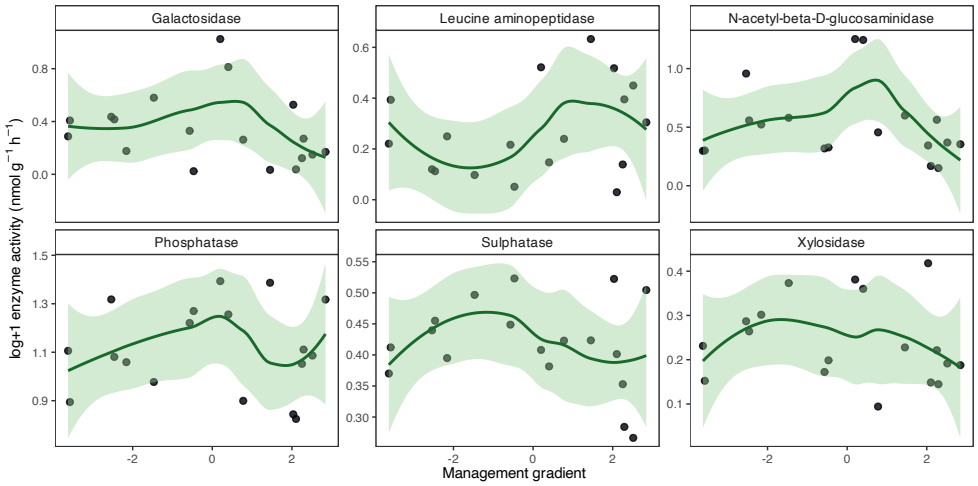


Figure S3.3: Results of Enzymatic activity assays, showing response of individual substrates (Galactosidase; 4-MUB-α-D-galactoside, Xylosidase; 4-MUB-β-D-xyloside, Phosphatase; 4-MUB - phosphate, Sulphatase; 4-MUB - sulfate, N-acetyl-β-glucosaminidase; 4-MUB-N-acetyl-β-glucosaminide and Leucine aminopeptisade; L-Leucine 7-amido-4-methylcoumarin) along management gradient

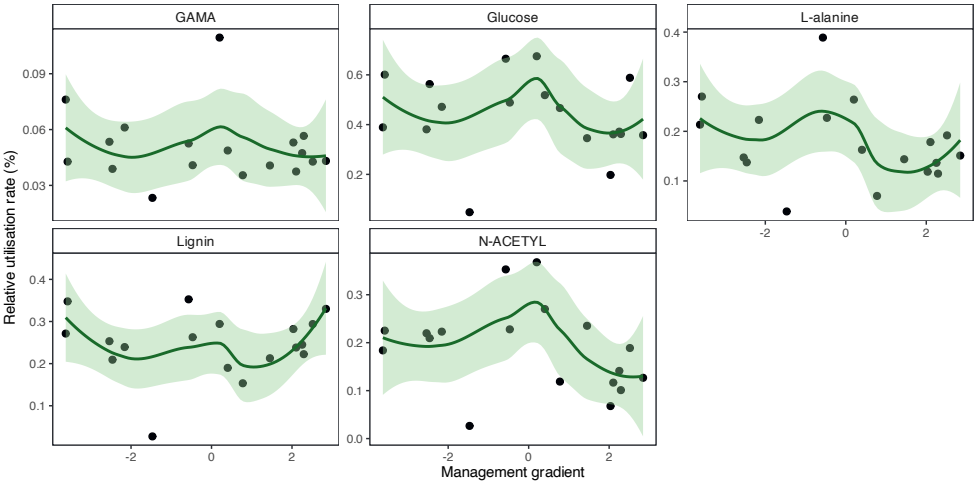


Figure S3.4: Results of MicroResp assay, showing response of individual substrates ((glucose (GLUC), lignin (LIG), N-acetyl glucosamine (N-ACETYL), gamma amino butyric acid (GAMA), alpha keto-glutaric acid (AKA), oxalix acid (OX), L-alanine (ALA), and miliq as a control) along management gradient

3.C Supplementary tables

Table S3.1: Information from farmer's surveys per field

Field	Age grassland (yr)	Age mgmt (yr)	Grass-cuttings/yr	Grazing /yr	LSU /ha	Chem. manure (kg N/ha)	Organic manure (m^3/ha)	Plant richness	Grass (%)	Legumes (%)	Forbs (%)
1	34	10	2	0	1	0	0	14.67	66.67	21.47	11.66
2	51	51	3	8	5	175	45	5.33	95.6	3.03	1.33
3	51	51	3	8	5	175	45	4.67	94.5	2.77	2.76
4	67	18	1	0	1	0	0	20.67	53.8	41.53	4.23
5	25	25	1	8	4	0	25	9	76.53	12.4	11.08
6	39	25	1	8	4	0	25	7.67	71.8	7.67	20.55
7	12	12	2	3.5	2	0	0	16	47.5	27.13	25.37
8	173	40	3	8	2	140	45	3.67	99.37	0.63	0
9	67	12	2	4	3	140	45	3.67	98.8	1.2	0
10	20	20	5	1	3	140	45	3.67	98.6	0.7	0.7
11	27	25	2	8	1	0	0	8.33	69.17	17.07	13.8
12	173	18	2	8	1	0	25	10.67	90.1	6.73	3.16
13	20	10	0	0	1	0	0	18.5	69	16.93	3.04
14	128	31	0	0	1	0	0	18.33	74.2	18.17	7.62
15	173	23	0	0	1	0	0	13.33	75.63	22.7	1.66
16	66	17	0	0	1	0	0	10.33	30.93	69.07	0
17	57	11	1	5	2	0	25	11.67	67.73	18.23	14.02
18	155	57	2	10	2	80	40	2.33	100	0	0

Table S3.2: Selected variables for management gradient, extracted from farmers surveys, including their descriptions, range and contributions to PCA axis (based on Euclidean distance matrix).

Number	Variable	Description	Range		
1	Age of grassland	The number of years it has been a grassland (i.e., time since establishment as grassland)	15–173 (year)	1.22	38.22
2	Age of management	The number of years it has been managed under the current management regime (i.e. how long the soil has been exposed to the given set of conditions)	10–57 (year)	12.68	16.92
3	Grass cuttings	The number of grass cutting events per year	0–5 (number/year)	13.12	11.70
4	Grazing	The number of months per year the grassland is grazed by cows	0–10 (number/year)	12.73	10.76
5	LSU/ha	Unit of Livestock per hectare, farm average	1–5 (LSU/ha)	13.58	15.32
6	Amount of chemical manure	The amount of chemical fertilizer in kg Nitrogen per hectare per year	0–175 (kg N/ha)	15.15	4.52
7	Amount of organic manure	The amount of organic manure in cubic meters per hectare per year	0–45 (m^3/ha)	16.30	0.22
8	Plant richness	The number of individual species found in each plot (2x2 m)	2–22 (nr. of species)	15.22	2.33

Table S3.3: Results from the Leave-one-variable out approach (Josse & Husson 2012). For each iteration, one management variable was removed from the dataset and the sPCA was recalculated. The resulting field scores were compared with those from the full model to evaluate the influence ofbut now th each variable on the ordination. Shown are the correlations between full-model and reduced-model scores for sPC1 and sPC2, as well as the mean positional shift of fields in the two-dimensional sPCA space. High correlations and low mean shifts indicate a robust management gradient that is not driven by any single variable.

Number	Variable left out	Correlation sPC1	Correlation sPC2	Mean shift
1	Age of grassland	1.00	0.56	0.86
2	Age of management	0.99	0.96	0.39
3	Grass cuttings	0.99	0.98	0.32
4	Grazing	0.99	0.98	0.33
5	LSU/ha	0.99	0.97	0.39
6	Amount of chemical manure	0.99	1.00	0.29
7	Amount of organic manure	1.00	1.00	0.24
8	Plant richness	0.995	1.00	0.22



Photograph taken at the author's family farm in Zeeland, the Netherlands.

4

Microbial scents: Soil microbial Volatile Organic Compounds (mVOCs) as biomarkers for grasslands across a land use gradient



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Abstract

Extensifying land use practices - i.e. decreasing management intensity - is pivotal to facilitate the transition towards sustainable agriculture in productive grasslands, as it promotes the enhancement of soil biotic communities that support important ecosystem services. To monitor these transitions effectively, improved inventories are needed to track changes in microbial communities. Microbial Volatile Organic Compounds (mVOCs) are potential biomarkers that can link soil community change to changes in management intensity (i.e. reducing land use intensity) and may provide important information on changes in soil processes in transitional productive grasslands. We investigated how variations on management intensity affects mVOC profiles in grassland soils through both abiotic and biotic factors and whether mVOCs can be linked to bacterial (16S) and fungal (ITS2) community composition. We measured mVOCs (GC-MS), microbial communities, and abiotic soil parameters (SOM, SOC, pH, bulk density, and nutrients) across eighteen grasslands with varying land use intensity. A total of 75 mVOCs were identified using the mVOC 4.0 library. Furthermore, we show that mVOC profiles and chemical classes differ across land use types - conventional, extensive, and semi-natural grasslands. Within these grasslands, only fungi showed distinct community compositions between land use intensity types, whereas there were no compositional differences of bacteria. Using Taxon Indicator Threshold Analysis (TITAN), we identified sixteen mVOC compounds that varied significantly along a gradient of management. These findings suggest that mVOCs can serve as biomarkers linking changes in land use intensity and soil microbial communities, although these relationships are complex in field conditions. We identified a set of mVOCs tied to changes in land-use extensification and highlighted their potential as indicators of soil microbial community turnover. mVOCs offer a valuable tool for monitoring land use transitions, and our results emphasize their role in integrating microbial community metrics and soil health indicators into land management strategies.

4.1 Introduction

The extensification - i.e. a decrease in management intensity - of current land use practices can enable the transition towards more sustainable use of productive agricultural grasslands (Cozim-Melges et al., 2024). Soil biotic communities are fundamental to nearly all soil processes and are believed to play a crucial role in sustaining soil productivity (van der Heijden et al., 2007). While the impacts of land use on soil biotic taxa vary (Cozim-Melges et al., 2024; Francioli et al., 2016; Lori et al., 2017), it is assumed that soil biotic communities are positively affected by land use extensification. Yet, measuring and monitoring the anticipated effects of such changes in land use practices on soil biota remains difficult for landowners, as exhaustive and often expensive analyses are needed to assess the composition of soil communities and their associated processes (Veum et al., 2017). Microbial Volatile Organic Compounds (mVOCs), VOCs produced by microbes, have been proposed as holistic indicators of soil biological composition (Brown et al., 2021). Nevertheless, the potential of mVOCs to underpin soil biotic community composition remains largely unexplored, notably in representative field settings (Insam & Seewald, 2010).

Soil microbes produce VOCs as a by-product of primary and secondary metabolism. mVOCs are known for their low molecular weight (<250 atomic mass unit) and high volatility, which diffuse through the non-saturated pore spaces of the soil matrix. Bacteria and fungi can also consume soil VOCs as a source of carbon (Albers et al., 2018; Rinnan & Albers, 2020). Hence, the diversity, abundance, and metabolic activity of the microbial community influences the soil mVOC fingerprint (Abis et al., 2020; Insam & Seewald, 2010). While not all mVOCs are unique to microbial kingdoms (Insam & Seewald, 2010; Tang et al., 2019), different microbial communities can create distinct mVOC profiles (Insam & Seewald, 2010). Several studies have shown that mVOC patterns can be used to distinguish between different phyla, strains or ecological functions, including saprotrophic or parasitic lifestyles (Choudoir et al., 2019; Guo et al., 2021). Data on VOC production by fungi and bacteria - mainly from in-vitro studies - have been aggregated into libraries, such as the mVOC Library 4.0 (Lemfack et al., 2018). These libraries allow the use of mVOCs for in situ detection of specific bacterial and fungal species. How well relationships between mVOC fingerprints and soil microbial communities hold in more complex field settings, however, hitherto remains underexplored.

Microbes in soils typically form complex communities characterized by various interactions, including microbe-plant, microbe-animal and microbe-microbe relationships. It is, for example, well-grounded in the literature that changes in the plant community are closely mirrored by shifts in the microbial community (Berg & Smalla, 2009; Schmid et al.

2021). These tight links collectively shape belowground processes essential for soil functioning. Additionally, interactions within the microbial community - often influenced indirectly by the composition of the plant community - can result in the context-specific production of various VOCs (Peñuelas et al., 2014; Schulz-Bohm et al., 2017). For instance, competition or antagonistic relationships can lead to the production of antifungal compounds that inhibit fungal growth (Peñuelas et al., 2014). VOCs can also be produced to influence certain microbial processes such as organic matter decomposition by inhibiting fungal laccase activity through bacterial VOCs (Mackie & Wheatley, 1999; Schöller et al., 2002). Therefore, the type of community and its metabolic activity may be expected to influence the mVOC profile.

In parallel to the biotic effects on mVOC production, soil abiotic conditions affect the characteristics of the mVOC profile. Several studies - largely under controlled circumstances - have demonstrated the rapid alteration of VOC profiles in response to changing soil, emphasizing the responsiveness of VOC production to environmental changes (Bastos & Magan, 2007; Brown et al., 2021; McNeal & Herbert, 2009). It has been suggested that litter decomposition forms a major part of the VOC emission profile (Leff & Fierer, 2008). Therefore, the input of organic matter could have a profound impact on the production of VOCs. As different kinds of organic matter are metabolized in different ways (due to variations in lability and quality), organic matter inputs could lead to distinct VOC production both in terms of compound composition and emission rates. For example, using organic amendments instead of inorganic fertilizer changes the quality and quantity of organic inputs, which could lead to an increase of saprotrophic and mycorrhizal fungi (Francioli et al., 2016). This alters the microbial community, its metabolic activity, and possibly the mVOC profile (Guo et al. 2021). In addition, environmental parameters such as pH, soil temperature and moisture may influence soil VOC production (Wester-Larsen et al., 2020).

Extrapolating links between abiotic and biotic conditions and mVOC production from controlled experiments to more complex systems like soils is challenging. Compared to isolated cultures, microbial taxa that grow in mixed communities exhibit a different metabolic activity resulting from the many facilitative and competitive interactions (Jean-Pierre et al., 2021). Furthermore, the specific genes that code for VOC production might be inactive in a monoculture but expressed when interacting with other organisms in soils (Brakhage, 2013), potentially resulting in different emission patterns. Microcosm studies may produce biased results, as they are typically conducted under controlled conditions or with soils that lack vegetation (e.g., Abis et al., 2020; Brown et al., 2021), limiting their ability to fully reflect natural ecosystem dynamics. Moreover, microcosms often experience edge effects, limited process transport, and restricted water flow, all of

which can alter microbial communities in ways that may not accurately reflect natural environments. Within productive grassland systems, soil microbial communities are directly and indirectly (through changing environmental parameters) influenced by land use practices (de Vries et al., 2006; Hartmann et al., 2015; Ollivier et al., 2011). Transitioning to more extensive land use practices - here specifically referring to reduced-intensity management, including the reduction of external inputs, rather than an increase in land area (van Grinsven et al., 2015) - can change specific soil chemical conditions and increase the diversity and activity of bacteria and fungi (Hartmann et al., 2015; Lupatini et al., 2017; Verbruggen et al., 2010). By switching from conventional to extensive management, the diversity and quantity of organic inputs change, which has been shown to shift microbial communities in the long term towards communities that are better adapted to decompose complex organic matter (Francioli et al., 2016; Hartmann et al., 2015). Consequently, to assess the link between mVOCs and soil microbial communities in productive grasslands, we need to urgently improve our understanding of the context dependent - biotic and abiotic - nature of mVOC production in natural soil environments.

Here, we assess to what extent variations in management intensity affects mVOC profiles and if this can be related to distinct microbial communities. To do so, we extracted soil mVOCs and their bacterial and fungal communities from productive grasslands that diverge in land use intensity types - conventional, extensive and semi-natural. We hypothesise that VOC profiles diverge between land use intensity types, and that these differences are driven by soil biotic and abiotic conditions. Additionally, we expect that the differences in VOC profiles will be, for a large part, caused by compounds related to organic matter decomposition and that the VOC profile of extensive grasslands will contain more compounds related to organic matter decomposition. We further hypothesize that variation in management intensity results in distinct microbial communities and that their community compositions would be strongly influenced by organic matter decomposition. As a final step, we relate changes in VOC profiles to shifts in microbial community compositions along a gradient of management. We expect that microbial genera can be linked to specific mVOCs. Last, we hypothesize that mVOC emissions depend on environmental parameters such as soil moisture and temperature.

4.2 Materials and methods

4.2.1 Study sites

Samples were taken from the Ooijpolder (51°51'39.8"N 5°54'29.5"E), situated along the river Waal near the city of Nijmegen, The Netherlands. This region is characterised by a long

history of agricultural use. Due to the proximity to the river, soils have undergone similar paedogenic processes. Hence, soils consist of river clay with a mixture of sand that varies between loamy sand and loamy clay sand (Rn95a, Dutch Soil Information System, Wageningen University). The area is characterized by a temperate-oceanic climate, with a mean annual rainfall of 832 mm and a mean temperature between 6.2 °C and 15.4 °C (March-September; Climate-data.org, Nijmegen 1992-2021).

We selected 18 permanent grasslands (age of management ≥ 10 yrs), located within a 12 km radius. These grasslands were selected on an a priori categorisation based on land use intensity type (Table 1). Hence, the 18 grasslands comprised conventional grasslands (n = 6), extensive grasslands (n = 6), and semi-natural grasslands (n = 6), ranging from most to least intensively managed grasslands. General management practices over the last decade were specified through questionnaires with the landowners (Table S1). Summarised, conventional grasslands are dominated by Lolium perenne L. and farmers use chemical fertiliser (type: non-disclosed) in combination with slurry to fertilise their fields. They further use pesticides for crop protection. The extensive grasslands are characterised by herb-rich swards of generally 6-16 species (predominantly Lolium Perenne L., Holcus Lanatus L., Bromus hordeaceus L., Trifolium repens L., Trifolium pratense L. and Taraxacum officinale F.H.Wigg.). No chemicals are used for management purposes. Fields are fertilised with organic manure. Both the conventional and extensive grasslands are cut and grazed. The semi-natural grasslands have previously been used for production but have been transformed to nature reserves (at least 10 years ago) and are now managed as such (i.e. no chemical inputs and no production, occasionally grazed). The vegetation is characterised by species-rich swards of 9-22 species (predominantly *Festuca rubra L., Agrostis stolonifera L., Poa trivialis L., Geranium dissectum L., Plantago lanceolata L. and Potentilla reptans L.*).

4.2.2 VOC sampling, analyses, and data processing

In October and November 2022, three 2 × 2 m plots were established in each grassland. This sampling allows to measure post-management effect at the shoulder of the growing season, while avoiding the immediate influence of the last manure application (15th of September). The placement of these plots was chosen randomly, but edge effects were avoided (i.e. plots were not allowed < 15 m from the grassland borders). Prior to soil sampling, soil VOCs were extracted from each plot using three individual soil probes (Wester-Larsen, 2020) - 25 cm long stainless-steel cylinders with a diameter of 3 cm, covered with 6 mm diameter holes (Markes International, Bridgend, UK) - placed in the upper left corner, centre and lower right corner of the plot (Fig. S4.1). After installation, the probes were left for at least 16 hours to avoid direct disturbance effects (following Wester-Larssen., 2020).

Table 4.1: Soil physicochemical properties across the three grassland management types. Values are mean ± standard deviation. Different superscript letters within a row indicate significant differences between management types.

	Type		
Variable	**Conventional**	**Extensive**	**Semi-natural**
Bulk density 0–10 cm	1.22 ± 0.10	1.14 ± 0.16	1.15 ± 0.015
Organic matter content (%)	5.87 ± 1.75	6.97 ± 1.60	7.46 ± 2.59
pH	6.3 ± 0.77^{b}	6.36 ± 0.97^{b}	7.39 ± 1.75^{a}
Al (kg/ha)	0.59 ± 0.28	0.83 ± 0.40	0.52 ± 6.34
Ca (kg/ha)	3536.98 ± 712.60	4457.87 ± 2218.76	4412.76 ± 1264
Fe (kg/ha)	0.46 ± 0.26^{ab}	0.57 ± 0.38^{a}	0.3 ± 0.19^{b}
K (kg/ha)	88.09 ± 66.05^{b}	239.19 ± 287.46^{a}	78.59 ± 98.35^{b}
Mn (kg/ha)	0.22 ± 0.23^{a}	0.28 ± 0.29^{a}	0.02 ± 0.01^{b}
P (kg/ha)	31.32 ± 30.90^{a}	23.54 ± 24.26^{ab}	10.44 ± 6.18^{b}
S (kg/ha)	32.43 ± 31.99^{a}	24.62 ± 24.89^{ab}	10.81 ± 6.40^{b}
Olsen P (kg/ha)	54.63 ± 19.68	76.81 ± 41.53	93.47 ± 0.01
NO_3^- (kg/ha)	142.12 ± 74.71	185.25 ± 102.39	167.27 ± 71.60
NH_4^+ (kg/ha)	0.67 ± 0.86^{b}	1.92 ± 1.69^{a}	0.09 ± 0.13^{c}
Mg (kg/ha)	639.92 ± 290.21^{b}	681.71 ± 238.10^{a}	295 ± 121.58^{c}
Field soil moisture (%)	31.09 ± 7.48	30.18 ± 8.44	29.62 ± 4.21
Field soil temperature (°C)	13.12 ± 0.81^{a}	12.82 ± 0.70^{b}	11.72 ± 0.85^{c}

Our own test to assess the effects of probe installation on the potential evacuation of VOC from our soils confirmed a recovery after 16 hrs (Fig. S4.2). Prior to VOC sampling, the air volume inside the probes were flushed (300 ml in total) with soil air, to ensure that accumulated VOCs due to installation were removed (Wester-Larsen et al., 2020). Subsequently, a conditioned thermal desorption tube (Tenax GR/Carbograph 5TD; Markes International, Bridgend, UK) was installed inside the probe. These tubes (conditioned before use) were selected for their capacity to capture a wide range of compounds and their hydrophobic properties. All three probes in each plot were connected to one pump for active air extraction at a 150 ml min-1 flow rate for 30 mins. VOC extraction was always conducted during the same time window (09:30-15:30) and under dry weather conditions to ensure consistency. Additionally, plots were sampled in a random order within each land use intensity type to avoid introducing treatment-related biases. After sampling, the thermal desorption tubes were capped and stored at 4 °C before measurement (within two to three days). Field soil moisture and temperature were recorded in three corners of the plots using a ML3 ThetaProbe (Delta-T services, UK) and a precision thermometer (Greisinger, GHT 175 PT-E). Across the sampling campaign, the field moisture and soil temperature were 28.8 ± 6.4 % and 12.2 ± 1.3 °C, respectively.

Analysis of VOCs was carried out on a TD-20 thermal desorption unit (Shimadzu, Kyoto,

Japan) coupled to a GC-MS (GCMS QP2010 Ultra, Shimadzu, Kyoto, Japan). Before analysis, thermal desorption tubes were purged with dry air to avoid potential interference by moisture. The desorption temperature was set to 280 °C and thermal desorption tubes were desorbed for 8 minutes under a helium flow (60 ml min-1). Desorbed VOCs were recollected on a Tenax TA focusing trap (Shimadzu, Kyoto, Japan) which was kept at -20 °C. After, the focusing trap was rapidly heated to 280°C for 5 minutes to release the VOCs which were then transferred onto a CP-Sil 19 CF capillary column (length: 25 m, internal diameter: 0.25 mm, film thickness: 1.2 µm; Agilent Technologies, Amstelveen, the Netherlands) at a split ratio of 5:1. The carrier gas was helium and the flow was set to 1.5 mL min-1. Instrumental settings were further adapted from Brown et al. (2021). Briefly, the column temperature was initially kept at 35 °C for 3 min, then increased to 100 °C at a rate of 10 °C min-1, then increased again to 220 °C at 20 °C min-1 (5 minutes hold time) and finally increased to 260 °C at 20 °C min-1 (hold time 10 minutes).

Chromatograms were processed using PARADISe v 6.0.1 (Quintanilla Casas et al, 2023). Shimadzu .qgd files were first converted to netCDF format using GCMS Solutions v2.72 (Shimadzu, Kyoto, Japan). After all GC-MS files were uploaded, auto-alignment was applied. After that, each peak interval was manually selected according to Quintanilla-Casas et al. (2023). Intervals were only selected if the peak occurred in at least three samples. After peak identification, models were built (10 iterations) after which the best fit was selected manually (> 95%). Selected peaks were exported and identified using NIST23 Mass Spectral Library (Shimadzu, Kyoto, Japan). Compound were only annotated if Match Quality was above 70% (NIST23 manual). Further data processing was done using an in-house MATLAB script (R2020a, The Mathworks, Inc., Natick, MA). Features were included when present in at least 50% of one of the field types. Peak areas of the remaining features were normalised to the total ion current (TIC). Further filtering steps were taken to remove VOCs without microbial origin. As plant roots are also known to emit VOCs (Insam & Seewald, 2010), this could potentially bias our result. Hence, we excluded all VOCs that have not been previously linked to microbial production (see results section for procedure). The CAS number of each feature was used in the mVOC 4.0 library to cross-reference all compounds, and only those defined as from microbial origin were included in further analysis (Lemfack et al., 2018). If the feature was not found in the library, or if it was indicated that the feature was not of microbial origin, the feature was removed. Classes were assigned to individual VOCs using PubChem (https://pubchem.ncbi.nlm.nih.gov/). mVOCs were categorised in classes: alcohols, aldehydes, alkanes, alkenes, alkynes, amides, aromatic hydrocarbons, carboxylic acids, esters, ethers, halogenated compounds, ketones, methylbenzenes, monoterpenes, sesquiterpenes, and sulphur compounds.

Soil sampling and biochemical analyses From each 2 × 2 m plot, soil bulk density was measured from soil cores (100 cc, Royal Eijkelkamp, Giesbeek, the Netherlands) that were taken at 0-5 cm and 5-10 cm depth. Additionally, twelve soil samples were collected (3 cm diameter, 0-25 cm depth) and bulked into one plot-specific composite sample. Composite soil samples were transported in coolers to the lab and stored at 4 °C. Within ten days, samples were homogenized and sieved (2 mm) to remove coarse materials and roots (Edwards et al. 2024; Rubin et al., 2013). From the sieved sample, a 1 g subsample was taken and stored at -20 °C for soil microbial community analysis. The remaining sample was used for chemical analyses.

Soil pH was measured using a NaCl extract (17.5 g of fresh soil in 50 ml 0.02 M NaCl, shaken for 2 h at 120 rpm). These extracts were filtered at 45 µm (Whatman paper), and analysed for nitrate (NO3-), ammonium (NH4+) and phosphate (PO42-) concentrations (Auto Analyser 3 system; Bran+Luebbe, Norderstedt, Germany), and other elements (e.g. Al, Ca, Fe, K, Mg, Mn, P, and S ; ICP-OES-iCAP 6000; Thermo Fisher Scientific Inc., Waltham, MA, USA). Plant available phosphorus (mg kg-1 dry soil) was determined following Olsen et al. (1954). A 20 g subsample of sieved soil was used to determine soil moisture content (105 C, for a minimum of 24h) and soil organic matter (Loss-on-ignition, 500 °C, 4h). For total carbon (°C) and nitrogen (N) content, sieved sample was first dried (40 °C) and ground, after which 20-30 mg was used for analysis (Elemental Analyser; NA1500, Carlo Erba, Thermo Scientific, U.S.A.). Plant species diversity was recorded per plot in May-July 2023, and the number of recorded individual species was used as a value of plant diversity.

Bacterial and fungal community compositions DNA was extracted from 0.25 g of sieved soil using the DNeasy PowerSoil Pro isolation kit (Qiagen) and cleaned up with the gDNA Clean Up kit (Qiagen) following the manufacturer's procedure and eluted in 50 µL of elution buffer. Extracted DNA was placed on dry ice before shipment to Novogene (Cambridge, UK), for metagenomic DNA amplification and sequencing. Extracted DNA was amplified for the V4 region of the 16S rRNA gene using specified primers 515F/806R (Caporaso et al., 2012) and for the ITS2 region of the ITS rRNA using primers ITS3-2024F/ ITS4-2409R (White et al., 1990). Polymerase Chain Reactions (PCR) were executed with 15 µL of Phusion® High - Fidelity PCR Master Mix (New England Biolabs), 2 µM of forward primers and roughly 10 ng template DNA. The PCR conditions consisted of an initial denaturation step of 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s and a final step of elongation at 72 °C for 30 s and 72 °C for 5 min. PCR products were quantified on 2

Microbial amplicons were sequenced using Illumina NovaSeq 6000 by with 250-bp paired-end reads (Novogene, Cambridge UK). After sequencing, barcodes and primers

were removed by Novogene. The trimmed data was then further processed in Dada2 (version 1.8) (Callahan et al., 2016) for both 16S and ITS gene amplicon reads, where sequences were further quality filtered and trimmed, with zero ambiguous bases allowed. For 16S truncation length was set at 218 for forward reads and 218 for reversed reads, for ITS2 a minimum length of 50 was used. To address the issue of binned quality scores of novaseq data in Dada2, we implemented a modified error rate estimation function, that iteratively calculates error rates for nucleotide transitions, employing local regression ('loess') to model error probabilities based on sequencing positions enforcing constraints to ensure error rates fall within defined limits. A maximum error estimate of 0.25 was used. For both 16S and ITS2, the consensus method was used to remove chimeras. For 16S the SILVA SSU database (version 138, 2019) was used (Quast et al., 2012). For ITS2 the UNITE fungal database (version 9.0) was used (Kõljalg et al., 2013; Nilson et al., 2019). From this, Amplicon Sequencing Variants (ASVs) tables were used for further analysis. Communities derived from 16S ASVs are referred to as 'bacterial' throughout, as archaeal sequences comprised a negligible proportion of the dataset (<0.5

4.2.3 Statistical analyses

All statistical analyses were performed in Rstudio v3.5.3 (R Core Team, 2021). For all analysis, the significance threshold was assumed at $p < 0.05$. To establish whether land use intensity types have different mVOC profiles, dissimilarities between total VOC profiles and VOC classes per land use intensity type were quantified using Euclidean distance and subsequently visualized using Principal Coordinate Analysis (PCoA) based on the resulting dissimilarity matrix. The impact of land use intensity on mVOC profiles was tested with a PERMANOVA analysis (Vegan package) on the Euclidean distance matrix. A posthoc pairwise PERMANOVA test with Bonferroni correction was performed to identify specific group differences. Homogeneity of multivariate dispersion was tested using the betadisper function to ensure assumptions of PERMANOVA are met. To test significant differences between mVOC classes, first the homogeneity of model residuals was checked (DHARMa package). If homogeneity did not significantly deviate, an ANOVA was used, and if significant, a Tukey's HSD posthoc test was used (stats package). If homogeneity did significantly deviate, a Kruskal-Wallis test was performed, and if significant a Dunn Test was used (FSA package). Significant differences between mVOC profiles were tested with a PERMANOVA and, if significant, further tested with a pairwise ANOVA (Vegan package).

To test if land use intensity types also lead to distinct soil microbial communities, bacterial and fungal ASVs were analysed within the Microeco package (Liu et al., 2021). To account for differences in sequencing depth between samples, we used the rarefy function

(sample size = 10000). For 16S, 16226 taxa were removed from the ASV table as the abundance was 0 after random subsampling. For ITS2, 7551 taxa were removed. A Bray-Curtis distance was used to quantify compositional dissimilarity. A Principal Coordinate Analysis (PCoA) was further used to visualise the bacterial and fungal community compositions per land use intensity type. Significant differences between land use intensity types were tested with a PERMANOVA, and if significantly different, further tested with a pairwise PERMANOVA (post-hoc) using a Bonferroni correction to account for multiple comparisons (vegan package, R). Assumptions of ANOVA were checked by testing the homogeneity of the model residuals. To measure to what extend the microbial communities were influenced by soil abiotic parameters, correlations were computed between soil chemical and physical parameters and the bacterial and fungal community compositions. As a measure for community composition, the most explaining PC axis from the bacterial and fungal PCoA were extracted.

Finally, we linked shifts in VOC profiles with shifts in microbial community composition along a gradient of management, using Taxon Indicator Threshold Analyses (TITAN) in the TITAN2 package (King & Baker, 2010). TITAN analysis allows the identification of indicative mVOCs and microbial taxa along an environmental gradient and visualises how their abundance changes along the gradient. Indicative features are selected based on purity and reliability. For the environmental gradient, we chose to use the variation in management intensity between individual fields based on the data from the questionnaires, scaling the fields from most to least intensively used grasslands. To create this management gradient, we first ordinated the fields using a Principal Component Analysis (PCA), using the prcomp function (Vegan package). This analysis used all numerical information from the questionnaires (Table S1) together with plant species diversity data at plot level. To avoid multi-collinearity, highly correlating variables (R > 0.8) within the same category (manure, crop protection, historical management) were removed and only one was selected for further analysis. The PCA showed a gradient of management intensity along the first PCA axis (explaining 60,56% of the variation, Fig. S4.3). This PC axis was therefore extracted and used as the environmental gradient (hereafter "management gradient") within the TITAN analysis. To test the relation between the management gradient and the microbial communities, the most-explaining PC axis from the bacterial and fungal PCoA were correlated with the gradient. To measure the effects of variations in management intensity on abiotic soil parameters, the gradient was also correlated with soil chemical and physical variables.

Furthermore, the gradient was used to identify indicative VOCs, bacterial and fungal genera with decreasing management intensity separately, using the TITAN analysis for all variables separately. For the bacterial and fungal genera, only the top 50% most abundant

genera were used, to optimize the TITAN analysis. After identifying indicative mVOCs and microbial taxa, their co-occurrence was measured using a correlation. These correlations were then cross referenced with the genera (or lower taxonomic levels if genera were not available) from the bacterial and fungal species recorded in the mVOC library (version 4.0).

To measure to what extent the mVOC profiles vary with environmental parameters, we computed the Pearson correlation coefficients and corresponding p-values (`rcorr` function (`Hmisc` package)) between soil chemical data and the mVOC classes that significantly differed between land use intensity type. mVOC classes were log-transformed prior to analysis and soil chemical data were scaled. To correct for multiple testing, Benjamini-Hochberg (BH) correction was applied to adjust p-values.

4.3 Results

4.3.1 mVOC profiles and classes

After filtering and the removal of non-microbial origin VOCs (using the mVOC library 4.0) we obtained a final list of 75 identified mVOC compounds (from a total of 120) of which 63 could be annotated with the NIST23 library. Across all land use intensity types, alkanes were hyperabundant, making up at least 53 % of the total composition. Unknown compounds also made up a large part of the composition (21%). Peak areas (TIC normalised) of the classes alkanes, aldehydes, carboxylic acids, esters, halogenated compounds, hydrocarbons, sesquiterpenes and sulphur compounds significantly differed between land use intensification types (Fig. 4.1; Table S4.2). Conventional grasslands showed the highest concentrations of aldehydes and sulphur compounds. Extensive grasslands showed the highest concentration for alkanes and carboxylic acids. Semi-natural grasslands had highest concentrations of esters, hydrocarbons, sesquiterpenes and halogenated compounds. As for the total mVOC profile, the composition of the profiles differed between land use intensity types (PERMANOVA, $F_{2,51} = 5.99$ $p = 0.001$), yet when comparing individual groups (pairwise PERMANOVA, Table S4.3) these differences could not be underpinned ($p \geq 0.05$). Based on the total VOC profile, we observed large variation within land use intensity types, with conventional and semi-natural grasslands being highly similar in mVOC profiles (Fig. 4.2). Although non-microbial VOCs were removed by using the mVOC library 4.0, some VOCs are known to be emitted by both plants and microbes (De Gouw et al., 1999; Delory et al., 2016; Mäki et al., 2019) which could potentially result in a slight overestimation of VOC production by microbes within our dataset. As we observed high similarity between conventional and semi-natural grassland mVOC profiles (Fig. 4.2), which differ most in plant community composition, we are confident that most of our VOCs are of microbial origin.

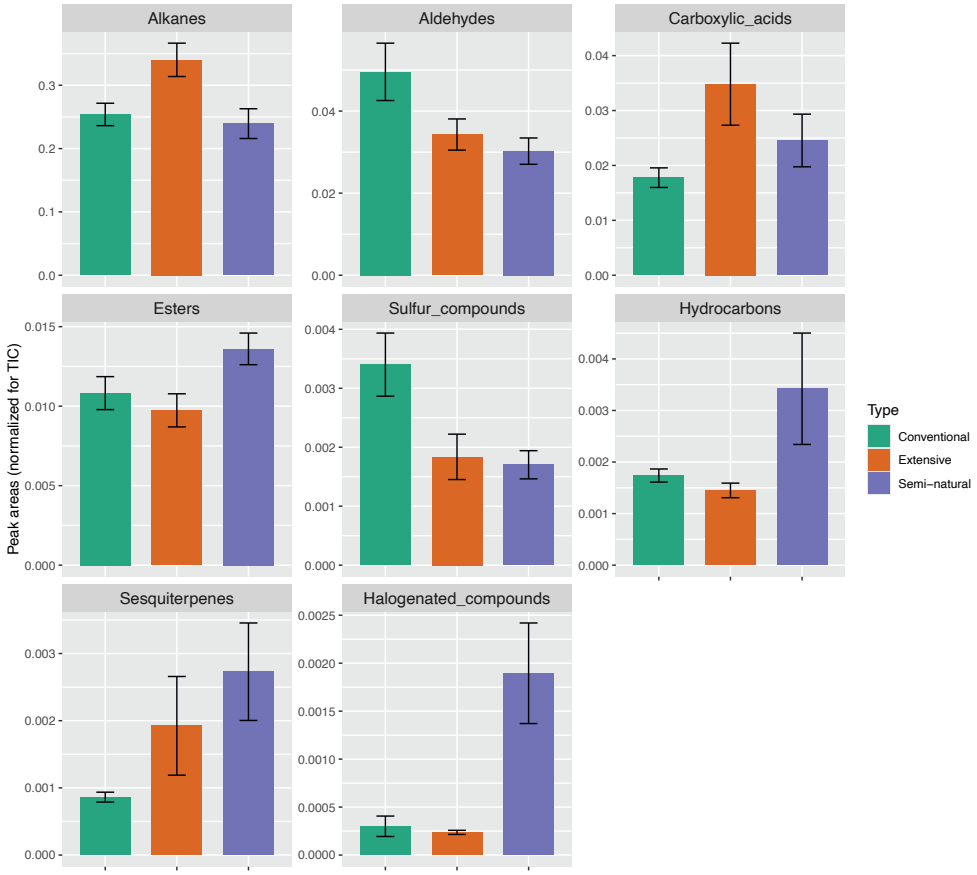


Figure 4.1: Average peak surface areas (normalised for Total Ion Current (TIC)) of the 75 mVOCs for all chemical classes, across three land use intensity types: conventional, extensive, and semi-natural grasslands. Grasslands were selected based on their management intensity, ranging from most to least intensively managed. Conventional grasslands are managed with external inputs (e.g. chemical fertilizer and pesticides), while extensive grasslands are managed with organic manure and no pesticides. Both grasslands are grazed and cut. Semi-natural grasslands, managed as nature reserves, receive no inputs and are sparsely grazed.

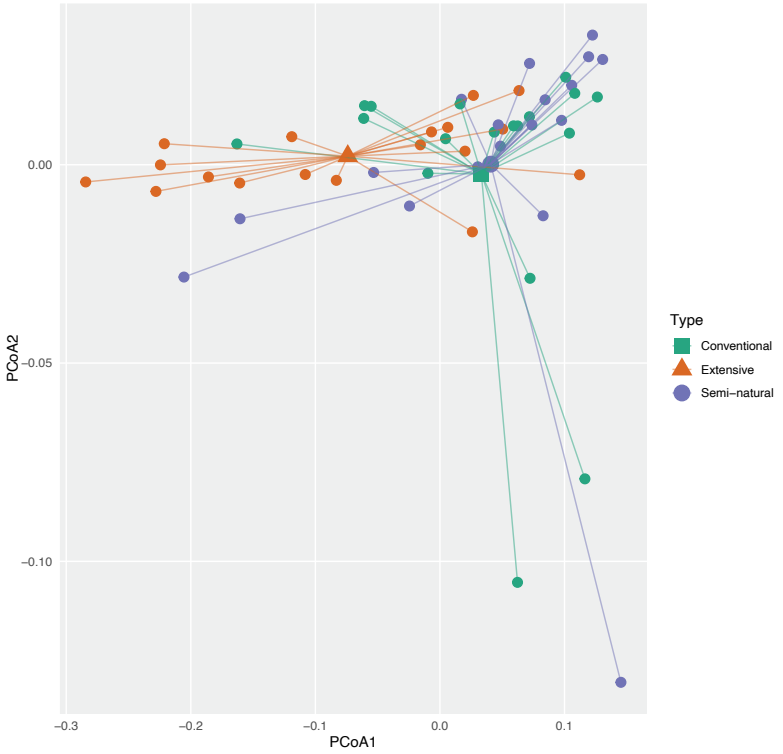


Figure 4.2: Principal Coordinate Analysis of VOC profile composition, based on Euclidean distance matrix. Plot shows distribution of mVOC profiles (based on peak surface area) per land use intensity type (named Type in the legend), with centroids showing mean of the group.

Bacterial and fungal communities Land use intensity affected bacterial community composition (PERMANOVA, $F_{2,51}$ = 2.06, p = 0.015), but this effect diminished when testing for differences between treatments (Pair-wise PERMANOVA, Table S4.4). When visualised, bacterial community composition was observed to have substantial overlap between land use intensity types (Fig. 4.3A). Interestingly, the effect of land use on fungal community composition was stronger, resulting in distinct clusters (PCoA, Fig. 4.3B and PERMANOVA, $F_{2,51}$ = 5.72, p = 0.001). Moreover, pair-wise analysis highlights all three types of land use intensity to differ in composition (Pair-wise PERMANOVA, Table S4.5). In similar fashion, the bacterial community composition did not show to be strongly affected by abiotic soil variables (Table 4.1), while fungal community composition did (Table S4.6).

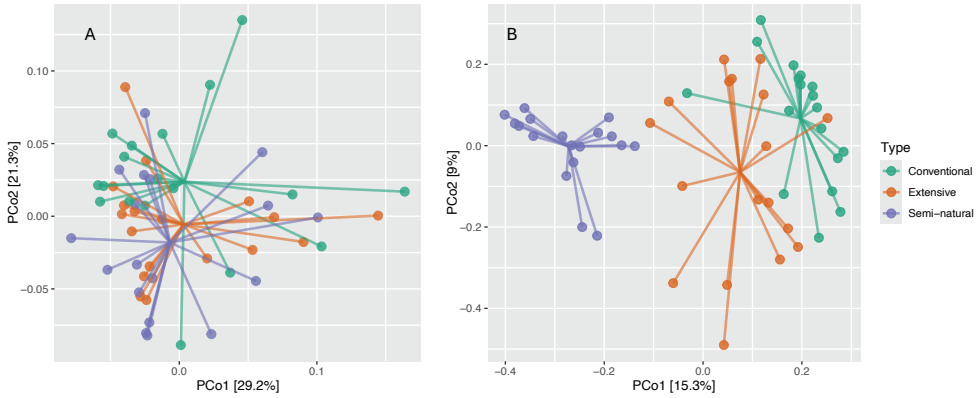


Figure 4.3: Principle Coordinate Analysis of A) bacterial (Class) and B) fungal (phylum) community composition based on relative abundance per land use intensity type (named Type in the legend), with centroids showing the mean of the groups.

4.3.2 Linking shifts in VOC profiles to shifts in microbial community compositions along a land use gradient

Sixteen indicative mVOCs were identified along the management gradient (Fig. 4.5). Nine of these compounds responded negatively to decreasing management intensity, which we will refer to as "-mVOCs." Conversely, seven compounds responded positively to decreasing management intensity, and we will refer to these as "+mVOCs.". For each indicative compound, a change point was identified, indicating the most probable gradient value at which each mVOC significantly changes. The change points for the -mVOCs tend to be on the lower end of the management gradient, whereas the +mVOCs typically have their change-point toward the higher end of the gradient, except for methyl acetate. This compound showed the highest overall z-score of 6.67, indicating that its abundance was significantly higher compared to the average abundance across the dataset (e.g. 6.67 standard deviations above the mean). Compared to the +mVOC, that showed well-defined change points, the density plots of the -mVOCs seemed to have less precise change points, except for dimethyl sulphide. Dimethyl sulphide had the highest z-score of the compounds that decreased (5.56). Among the -mVOCs, the chemical class of aldehydes was most present (heptanal, hexanal, octanal, and nonanal).

The variation within the bacterial community did not show a strong correlation with the land use extensification gradient (R^2 = 0.07, Fig. S4.4), while the fungal community did (R^2 = 0.68, Fig. 4.4). For most of the environmental variables, there was a strong significant correlation with the gradient (Table S4.7). Only for parameters related to nitrogen, aluminium, plant-available Phosphorus and soil bulk density, the correlation was not significant.

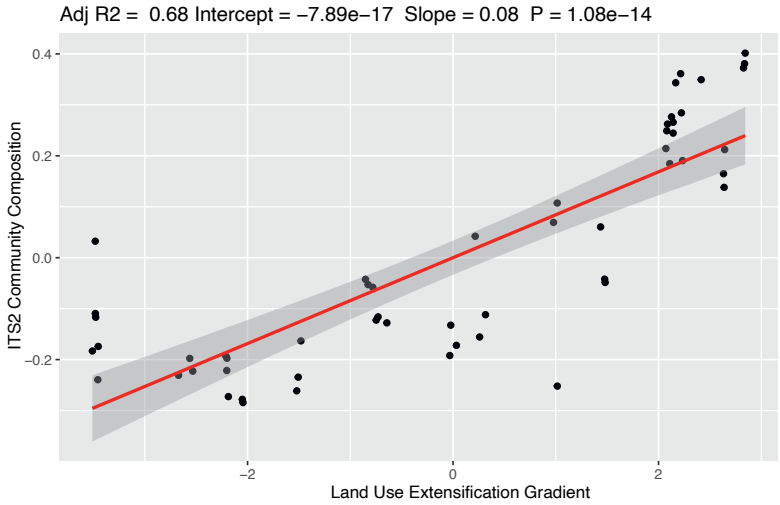


Figure 4.4: Relationship between ITS2 community composition (relative abundance) and management gradient (PC1 values). The shaded area represents the 95% confidence interval of the regression.

The indicative bacterial genera showed a more even distribution of positively and negatively responding genera (16 versus 21, 37 in total), while indicative fungal genera were identified more often as positive (27 versus six, 33 in total) (Fig. S4.5A, Fig. S4.5B). Fungal genera exhibited stronger correlations with the indicative mVOCs, both positively (R > 0.40) and negatively (R < -0.40), compared to bacterial genera (Fig. S4.6, Fig. S4.7). When trying to cross-reference indicative mVOCs and the indicative bacterial and fungal genera with their co-occurrence known from literature (using the mVOC library 4.0), only one bacterial genus (Streptomyces as producers of beta-pinene, R = -0.03) and one fungal genus (Cladosporium as producers of Methyl Benzoate, R = -0.07) was found (Table S4.8).

Covariation of soil chemical variables with significant VOC classes Between land use intensity types, average field soil temperature, pH, plant species richness, and NH4+ significantly differed, while organic matter content, average field soil moisture, bulk density and NO3- did not. For each soil chemical variable, covariations with peak areas of significant mVOC classes were mixed, showing neither exclusively negative nor exclusively positive relationships (Table 4.2). In addition, none of the variables showed significant regressions with all classes. Average pH had the most significant correlations with VOC classes, while NO3- and plant available phosphate (Olsen P (kg/ha)) did not show any significant relationships. Also, aldehydes and carboxylic acids did not significantly correlate with any soil chemical variable, while alkanes had most significant correlations.

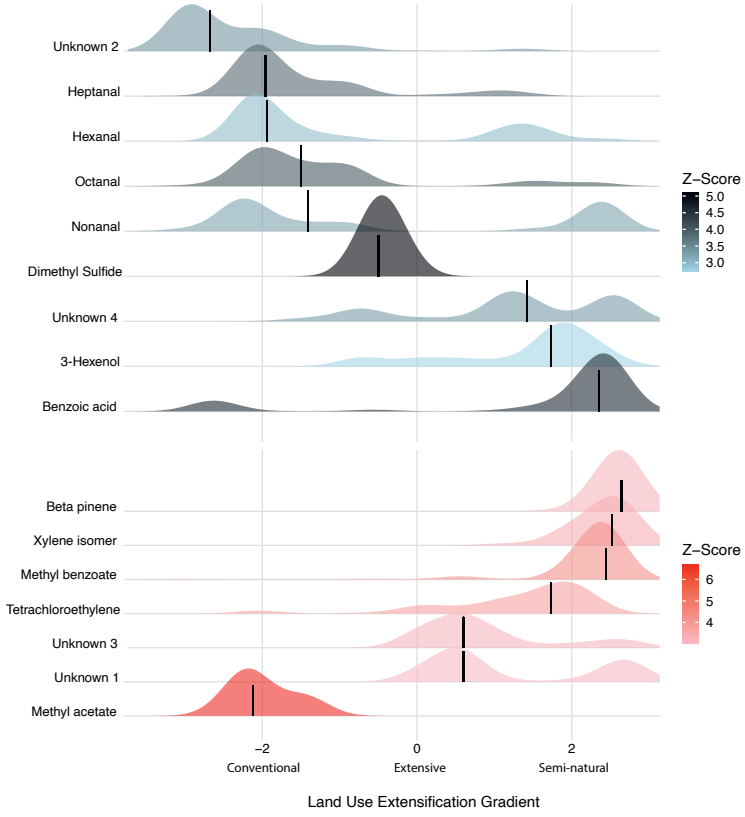


Figure 4.5: Indicative mVOCs along a gradient of management, as identified with a TITAN analysis (King & Baker, 2010). mVOCs names are presented on the y-axis, with their corresponding change point, as the probability density functions of the bootstrapped replicate. The mVOCs in grey show the compounds that decrease in abundance (-mVOCs) along the gradient, while the mVOCs in red show the compounds that increase in abundance (+mVOCs) along the gradient. The vertical line represents the median change point, indicating the most probable land use extensification value at which each mVOC significantly changes. Z-score shows the standardised measure of deviation from the mean abundance across all samples.

4.4 Discussion

Understanding how extensification - i.e. a decrease in land use intensity - affect soil microbial communities is essential as the latter drives important soil processes. The aim of this research was to identify the effects of extensification on mVOC profiles and to explore their use as biomarkers for microbial community composition. Our analysis evidenced that mVOC profiles differed between the three types of land use intensity. Moreover, we identified sixteen compounds that are indicative for the gradient of management.

Table 4.2: Correlations between soil physicochemical variables and mVOC compound classes. Asterisks (*) indicate significant correlations ($p < 0.05$).

	Alkanes	Aldehydes	Carboxylic acids	Hydro-carbons	Esters	Halogenated compounds	Sulphur compounds	Sesqui-terpenes
Organic matter content (%)	0.36*	-0.28	0.11	-0.26	-0.24	0.23	-0.21	-0.27
Field soil moisture (%)	0.03	-0.22	-0.06	0.06	0.09	0.32	0.43*	-0.04
Field soil temperature (°C)	0.29	0.07	-0.22	-0.34	-0.23	-0.19	0.51*	-0.27
pH	-0.43*	-0.06	-8.8×10^{-3}	0.54*	0.55*	0.39*	0.20	0.45*
Bulk density (0–10 cm)	-0.43*	-0.02	-0.01	0.24	0.29	-0.03	0.32	0.03
Plant richness (nr. of species)	-0.01	-0.19	0.30	0.22	0.20	0.35*	-0.36*	0.26
NO_3^- (kg/ha)	-0.10	-0.13	-0.10	0.19	0.11	0.20	0.03	0.05
NH_4^+ (kg/ha)	0.58*	0.17	0.03	-0.41*	-0.49*	-0.34	-0.19	-0.27
Olsen P (kg/ha)	-0.22	-0.24	-0.01	0.03	0.11	0.24	0.10	0.01

Interestingly, we found relations between the VOC profile and microbial community composition, yet these did not correspond to relationships that are assumed in the mVOC database (4.0) which are based on controlled in-vitro studies. Apart from relationships with microbial communities, mVOC classes also significantly covaried with soil chemical parameters, especially soil pH. These results highlight that the microbial-related VOC profiles from soils are strongly context dependent and more difficult to directly link to microbial community composition. Additionally, potential plant root VOC emissions, which may not have been entirely excluded, could influence our findings. Nevertheless, our results show that mVOCs can be used as biomarkers for changes in management intensity.

4.4.1 mVOC profiles as proxies for land use intensity type

We hypothesized that VOC profiles diverge between land use intensification types due to differences in soil biotic and abiotic conditions. Our work did show differences in mVOC emission between different types of land use intensity. Based on chemical classes, there was a significant difference in the peak areas of alkanes, aldehydes, carboxylic acids, esters, sulphur compounds, hydrocarbons, sesquiterpenes and halogenated compounds between land use intensity types (Fig. 4.1). Alkanes were the most abundant class for all types, particularly within extensive grasslands. They are predominantly emitted in soil by both bacteria and fungi, such as Streptomyces, Cyanobacteria and Pseudomonas (Wenke et al., 2012). Notably, their production is associated with decarboxylation, a key process in the decomposition of organic matter under aerobic soil conditions (Peñuelas et al., 2014; Yan et al., 1996). Also, the profile based on mVOC emissions (Fig. 4.2) showed a divergence (although not significant after adjusting for multiple comparison) between extensive grasslands and conventional and semi-natural grasslands.

In our study, extensive grasslands received less solid manure and different inputs of organic matter (straw rich stable manure or slurry), as compared to conventional (predominantly mineral fertilizer in combination with slurry) and semi-natural grasslands (none) (Table S4.1). In addition, organic matter content increased along the management gradient (Table S7). While not tested specifically, consequential apparent differences in organic matter dynamics may have altered metabolic activity, microbial abundance and community composition, and thus could potentially explain the differences in mVOC profiles (Francoli et al., 2016; Taylor et al., 2014).

The overall mVOC profile composition (Fig. 4.2) varies between land use intensification types based on all individual mVOCs present. However, this difference disappeared when comparing individual groups, likely caused by the large amount of variation within land use intensity types. Increasing the sample size might amplify differences between land use intensity types. Alternatively, it is possible that many mVOCs in soils lack discriminatory power, suggesting we should focus on those that do. A TITAN analysis does so, by identifying indicative compounds, based on reliability and purity of those compounds (Baker & King, 2019).

We identified sixteen compounds along a management gradient (Fig. 4.5). Although alkanes were significantly different and most abundant between land use intensity types, along the management gradient (showing an decrease in management intensity) not alkanes but aldehydes (heptanal, hexanal, octanal and nonanal) were among the most important indicative mVOCs. All four compounds decreased in abundance with a decrease in management intensity. This suggest that aldehydes are more sensitive to gradual changes

in management intensity, compared to alkanes. In general, fungal VOC emission profiles are often dominated by aldehydes (Peñuelas et al., 2014). They have been previously found in soil microcosm studies, where they showed to be significantly different between soils under different environmental treatments (Asensio et al., 2007; McNeal & Herbert, 2009). Specifically, McNeal & Herbert (2009) showed that heptanal and hexanal were significantly different between treatments with varying types of substrate amendments, which could indicate their sensitivity to changes in organic matter decomposition. In contrast, studies have shown that aldehydes are also produced by plants after tissue damage (de Gouw et al. 1999; Delory et al. 2016), raising the possibility that the aldehyde production could be related to the installation of the probes (e.g. vegetational damage belowground). However, if this effect would be significant, it will be comparable across fields. Additionally, the 16-hrs gap should be sufficient to allow for most emissions caused by disturbance to stabilise (Fig. S4.2). Moreover, no significant correlation was found between aldehyde emissions and plant diversity (Table 4.2), further suggesting that the variation in aldehyde emissions was likely caused by for the larger part by belowground microbial communities.

Most indicative mVOCs (including aldehydes) in our study tended to decrease in abundance with decreasing management intensity. This suggests that either there is an overall decrease in the production of these compounds or an increase in the consumption of these compounds - e.g. though changes in the microbial community - when management intensity changes. On the one hand, changes in organic matter input between land use intensity types could change VOC emission profiles, as described above. VOC emissions tend to correlate with substrate quality (Brown et al., 2021; Mancuso et al., 2015; McNeal & Herbert, 2009), which could be driven by microbial communities that are specific for these different inputs (Stahl & Parkin, 1996). On the other hand, a decrease in management intensity could also result in an increase of VOC-consuming species or activity, leading to an overall decrease in the net concentration of the specific mVOCs (Abis et al., 2020; Jiao et al., 2023). However, almost all mVOCs did not show well-defined change points (Fig. 4.5), which suggest that they are indicative of change in management intensity, but not restricted to a type of land use.

A compound that did have a well-defined change point was Dimethyl Sulphide (DMS), indicating its potential as indicator compound. It had the highest Z-score of the -mVOCs and correlated most with the fungal genera Neoascochyta (R = 0.42, Fig. S4.7). Neoascochyta also decreased with increasing land use extensification, around the same change point as DMS. Although this underlines their potential relation, we are not aware of demonstrated DMS production by Neoascochyta. DMS is a compound that is produced during the microbial catabolism of sulphur-containing amino acids (Schäfer et al., 2010). Although it is

known to be an important intermediate molecule in the C and S cycle, the origin of its production is still not clear (Mancuso et al., 2015). However, it has previously been found in fungistatic soils, which could indicate that when shifting from extensive to semi-natural grasslands in our study, soil microbial communities are less investing in the inhibition of fungal growth (De Boer et al., 2003; Garbeva et al., 2011).

4.4.2 Fungal communities as drivers for mVOC profiles

We characterized the microbial communities of the samples and linked these with their corresponding mVOC profile. While the bacterial communities did not show distinct clusters (Fig. 4.3A), fungal communities clearly separated (Fig. 4.3B). This contrasting response of bacteria and fungi is contradictory to our hypothesis and to previous studies, which found that both bacterial and fungal communities were affected by land use intensity (Hartmann et al., 2015; Lupatini et al., 2017; van Rijssel et al., 2022). In our study, it was clear that management practices had an effect on shaping these fungal community compositions, as fungal community composition was strongly correlated to the management gradient, while bacterial community composition did not. It is possible that fungal community build-up better reflects the effects of long-term management practices, because their hyphal networks need more time to develop. Bacteria on the other hand could be more responsive to short-term effects, as their development is more dynamic (Six et al., 2006).

The contradictory response between bacteria and fungi was also evident when considering the relationships of microbial community composition and soil abiotic parameters. Bacterial community composition did not seem to correlate significantly with almost all variables (except for manganese), whereas fungal community composition did significantly correlate with most of the variables. Notably, there was a significant correlation with soil organic carbon content (%) (R = 0.4) but not with organic matter content, (%) (RT = 0.25), in contrary to our hypothesis. This result could indicate that the fungal community is more strongly influenced by the more stable forms of carbon, which are stabilized as soil organic carbon, rather than labile organic matter inputs. As a decrease in management intensity in this study involves changes in the quality of organic matter inputs (Table S1), this could promote the growth of fungal communities that are better adapted to more stable forms of carbon. This observation aligns with the findings of van Rijssel et al. (2022).

Because only the fungal communities showed a differentiation between land use intensity types, we suggest that the differences measured in mVOC profiles are a result of differences in fungal community compositions. Although not measured in soils, Calla-Quispe et al. (2020) suggested that the emission profile from lichen thalli would be mainly pro-

duced by fungal species, as fungi are able to produce a wide variety of specialised compounds. However, the species that were indicative along the management gradient were mostly decreasing with decreasing management intensity (Fig. S4.5B). For the fungal communities this is surprising, because previous studies (Francioli et al., 2016; Hartmann et al., 2015) suggest that with decreasing management intensity (e.g. extensification), fungal communities become more complex and therefore show an increase in relative abundance of species. Almost all genera that were indicative belonged to the phylum Ascomycota, which are known to be generalists (Egidi et al., 2019). In a study by Abis et al. (2020) Ascomycota did not have many strong correlations with VOC emissions rates from inoculated soils. However, in our study it seems that some genera within this phylum highly correlate, while others do not. This suggests that the variability within a phylum in mVOC production, may be genus specific.

Besides showing an overall decrease in abundance of indicative fungal genera's, these genera also exhibited stronger negative correlations with the indicative mVOCs than bacterial genera did (Fig. S4.6). The overall decrease in indicative mVOCs, combined with stronger negative correlations between fungal genera and these compounds, suggest that fungi may be more effective at removing VOCs. This stronger efficiency has also been proven by Estrada et al. (2013) who compared fungal biofilters with bacterial biofilters. This can also be related back to the decrease in most of the indicative mVOCs along the gradient (Fig. 4.5), as with a decrease in management intensity, fungal biomass tends to increase as well (de Vries et al., 2006). Therefore, with decreasing management intensity, more mVOCs may be consumed, resulting in a decrease of many mVOCs along the gradient, as is seen with most of the indicative mVOCs. Conversely, since the overall mVOC profile did not display distinct clusters like the fungal communities did, it is possible that the microbial biomass in these grasslands is predominantly composed of bacterial biomass, and therefore outweighs the fungal mVOC production within the total emission profile.

The relationships between indicative mVOCs and fungal genera did not align with previous established relationships, based on existing in-vitro studies (Table S8). Using the mVOC library (4.0, Lemfack et al. (2017), we searched for known producers of the indicative mVOCs. However, this search only revealed an overlap between indicative mVOCs and microbial genera for beta-pinene and methyl benzoate, both identified as increasers in the TITAN analysis. This contradicts our hypothesis, which anticipated that the relationships between the specific mVOCs and microbial genera in this study would mirror those described in the mVOC library. Moreover, the mVOC library is mainly build upon in-vitro studies, as identifying producers of specific mVOCs is more obvious when cultivating a ho-

mogeneous culture under controlled circumstances.

This also introduces some drawbacks. Studies have already underlined that medium composition and microbial interactions (in the case of simple synthetic microbial communities) can have a strong influence on the production of mVOCs (Lazazzara et al., 2017; Tyc et al., 2017; Wester-Larsen et al., 2020). As our study was conducted in a more complex environment (e.g. the soil matrix), relations become less obvious, but also better reflect the natural mVOC production in soils. Nevertheless, we still found sixteen indicative mVOCs, which can now be used in future field studies (Downs, 2006). Although there were no evident links between the indicative microbial genera and mVOCs, there were strong positive and negative correlations found for both the negative and positive responding microbial taxa when correlating these with the indicative mVOCs (Fig. S4.6 & S4.7). This suggests that although there are possible producers of these indicative mVOCs present, they are not known yet.

4.4.3 Soil pH determines fungal community composition and mVOC profiles

Our approach also allowed us to study how environmental parameters influenced mVOC profiles. In our study, environmental variables covaried with mVOC production, which is in support of our hypothesis (Table 2). Some relations align with established findings on soil microbial activity and environmental parameters, such as the positive correlation between sulphur compounds and field soil moisture: This may be indicative of anaerobic conditions favouring sulphate-reducing bacteria that produce sulphur-containing VOCs (Lomans et al., 2002). Surprisingly, the parameter with the most significant correlations, pH, was also the parameter that correlated most strongly with the fungal community composition. This indicates that pH could by the driving process behind VOC production by fungi. pH significantly impacts microbial communities overall, with high pH specifically associated with fungistasis and increased ammonia production.

However, there was not one variable that showed exclusively positive or negative relations with chemical classes. This means that chemical classes show variable responses to environmental variables. This in contrast to the findings by Wester-Larsen et al. (2020) and Jiao et al. (2023) who found strong positive relations between VOC concentrations and soil moisture and temperature. Soil moisture and temperature are known to have a significant effect on microbial activity (Blagodatskaya & Kuzyakov, 2013). Yet, both mVOC production and consumption may be accelerated with higher temperatures, leading to different net concentrations depending on the site, microbial communities and environmental factors.

The diverse responses observed across different VOC classes in this study empha-

sizes the complexity and variability that is inherent to VOC emissions. These findings underscore the necessity of including soil properties alongside VOC measurements, to accurately interpret and monitor VOC profiles in grassland ecosystems. To determine how soil chemical variables influence VOC emissions, conducting measurement of VOC profiles over extended periods is essential in future research, rather than relying on single-point measurements.

4.4.4 Measuring in-situ soil mVOC profiles

Not many studies have measured mVOCs in complex samples (e.g. soil) and in combination with the characterization of the microbial community (Leff & Fierer, 2008; Sillo et al., 2024). Previous studies have attempted to exclude plant root emissions from soil VOC profiles by using root-inhibiting meshes in field-settings (e.g., Mäki et al., 2017) or through pretreatment of soils in more controlled experiments (e.g. Abis et al., 2020; Brown et al., 2021). In this field study, minimising disturbance was prioritized over potentially overestimating microbial VOCs due to root emissions, as plant roots are a natural component of the field system. Methods such as mesh installation or soil sieving can significantly disrupt microbial communities, potentially altering mVOC profiles by releasing VOCs from damaged fungal hyphae, altering microbial community composition or causing labile carbon fluxes that stimulate organic matter decomposition (Mäki, 2019). Such disturbances may lead to mVOC production that do not reflect natural soil conditions.

By studying VOCs in an intact soil ecosystem, our approach allowed us to observe how field conditions shape mVOC profiles. Although this study measured mVOCs together with the microbial community composition, it did not explore the relationship with community functioning. Soil microbial communities are known to drive important soil functions, yet functional redundancy in both microbial and plant communities can be high, as shown by other studies (Chen et al., 2024; Robroek et al., 2017). Because mVOC profiles are partly driven by the functional capacity of these communities (e.g., metabolic activity), functional redundancy could potentially cause overlap in mVOC profiles. Therefore, this should be further investigated in future studies.

4.4.5 Conclusion

The transition towards land use extensification in productive grasslands could be eased with new biomarkers that are able to monitor changes in soil microbial communities. Here, we measure the potential of microbial volatile organic compounds as such biomarkers. Within grassland soils under differing management, we found 75 mVOCs from a total of 120

VOCs. While mVOC profiles showed high overlap between land use intensity types, there were significant differences between peak surface areas for eight mVOC chemical classes among these types. Alkanes were the most abundant chemical class, being the highest in extensive grasslands, indicating the role of organic matter decomposition in driving differences in mVOC production.

Some mVOCs showed to be better indicators of change than others. Using a TITAN analysis, we identified sixteen mVOC compounds that showed to be indicative of an increase in land use extensification. Specifically, aldehydes (including heptanal, hexanal, octanal and nonanal) showed to be sensitive to changes in management regimes, responding negatively to an increase in extensification.

The difference in mVOC profiles could be driven by distinct fungal communities. While bacterial communities showed overlap between land use intensity types, fungal communities showed clear distinctions between types. However, trying to relate specific microbial genera's with mVOCs based on previous in-vitro experiments, turned out to be difficult. This could be due to the high amount of complexity brought upon by a field-study, such as plant root emissions. Plant roots are known to emit VOCs belowground, and this could potentially result in an overestimation of mVOC production within this study. Yet, plant root emissions are inherently part of the VOC profile, and removing plant roots would hinder the holistic view of mVOCs in the natural field context.

In addition to these biotic parameters, abiotic soil variables also showed to have an effect. In particular, soil pH showed to have the significant effect, which also correlated strongly with fungal community composition. However, none of the mVOC classes showed exclusively positive or negative relations with soil abiotic parameters. Overall, this study emphasized the complexity of soil mVOC emissions and their relationship with soil biotic and abiotic parameters. We therefore advocate for further research, particularly field-based studies, to better understand these dynamics and improve the use of mVOCs as reliable indicators of soil microbial communities and activity in varying land use contexts.

4.4.6 Acknowledgements

We thank Jan Peter Mayser from Markes Internation (UK) for his help in developing a VOC sampling method in soils. We much valued the help of Annemieke Tiekstra, Peter Cruijsen, Roy Peters and Niels Wagemaker with sample collection and the processing of soil samples. We also are appreciative to all the farmers for allowing us to sample from their soils. The imec team, notably Francesca Leonardi, Burçu Celikkol and Marcel Zevenbergen, provided for valuable advice, guidance and comments on early drafts of this work. Last, two

anonymous reviewers provided constructive comments that helped with improving the narrative and context of our work.

4.4.7 Data availability statement

Data available from the DANS-EASY data repository: https://doi.org/10.17026/LS/32VHKC

Chapter appendix

4.A Supplementary figures

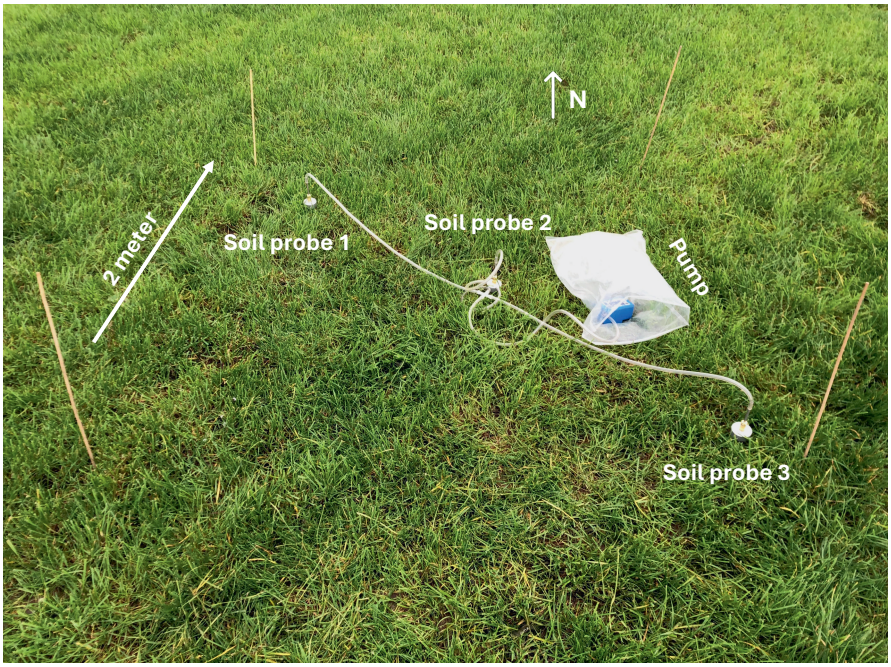


Figure S4.1: Field sampling set-up for mVOC extraction, showing three soil probes within a 2 x 2 m plot attached to a pump, in a conventional grassland.

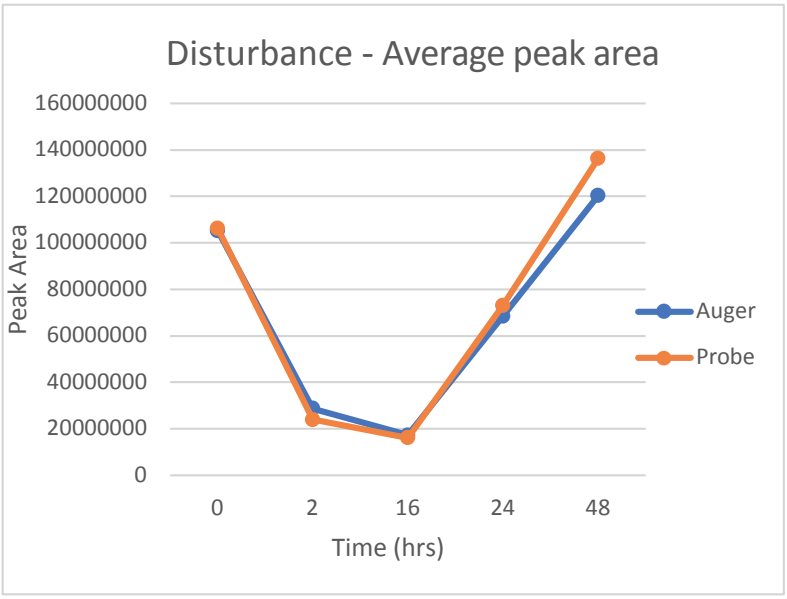


Figure S4.2: Graph showing the average peak area (n=5) right after installment of the probe (Time = 0) and 16 hours after (Boone et al. unpublished). Probes were installed at five random locations in a grassland situated in the Ooijpolder Nijmegen (April, 2023). The graph indicates that the disturbance caused by the installation of the probe settles down at 16 hours after placement.

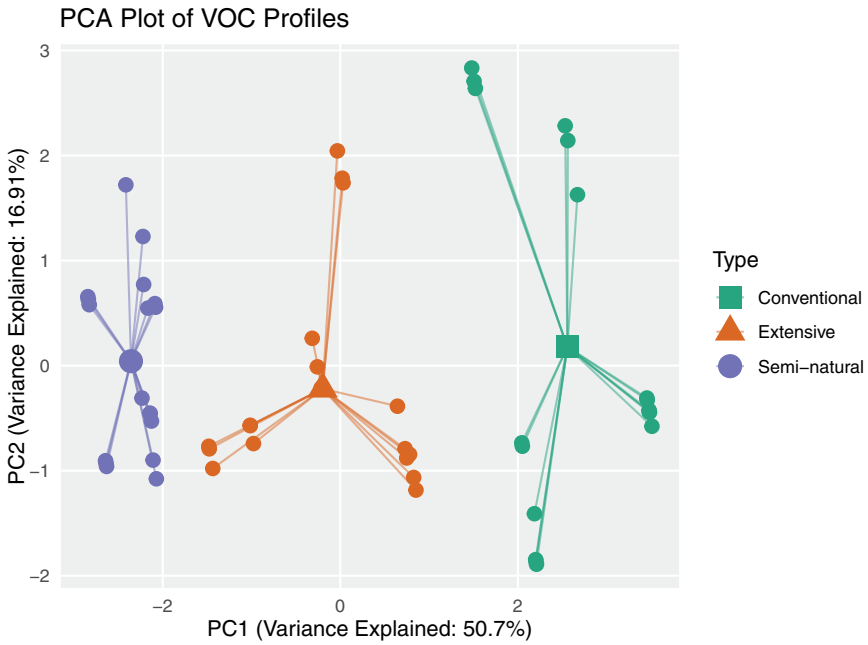


Figure S4.3: Principle Component Analysis created with Euclidean distance matrix based on numerical data from questionnaires

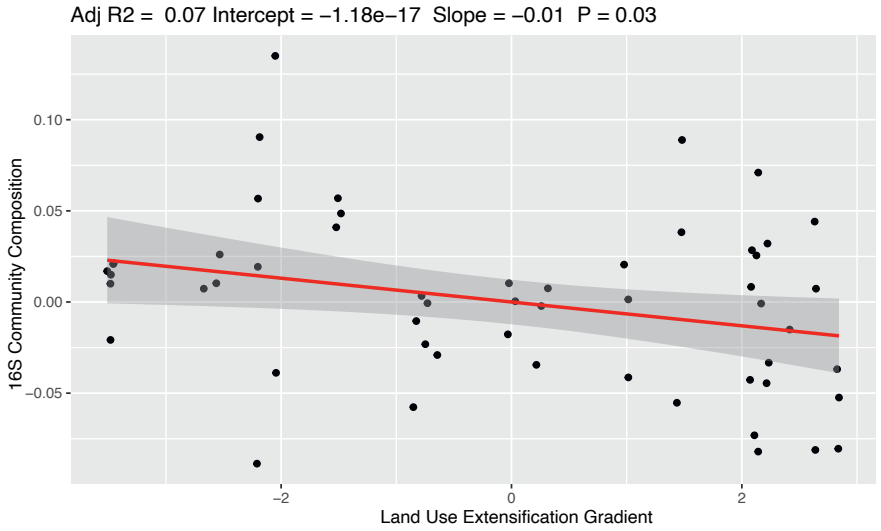


Figure S4.4: Relationship between 16S community composition (relative abundance) and management gradient (PC1 values). The shaded area represents the 95% confidence interval of the regression.

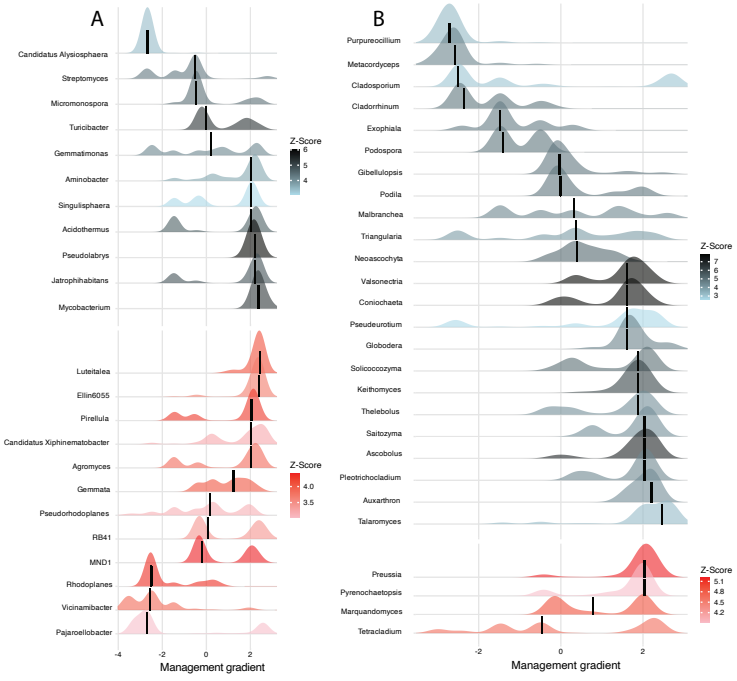


Figure S4.5: Indicative bacterial (A) and fungal (B) genera along a gradient of management (decreasing in management intensity), as identified by TITAN analysis. Bacterial and fungal genera are presented on the y-axis, with their corresponding change point, as the probability density functions of the bootstrapped replicate. The vertical line represents the median change point, indicating the most probable gradient value at which each genera significantly changes. Z-score shows the standardised measure of deviation from the mean abundance across all samples.

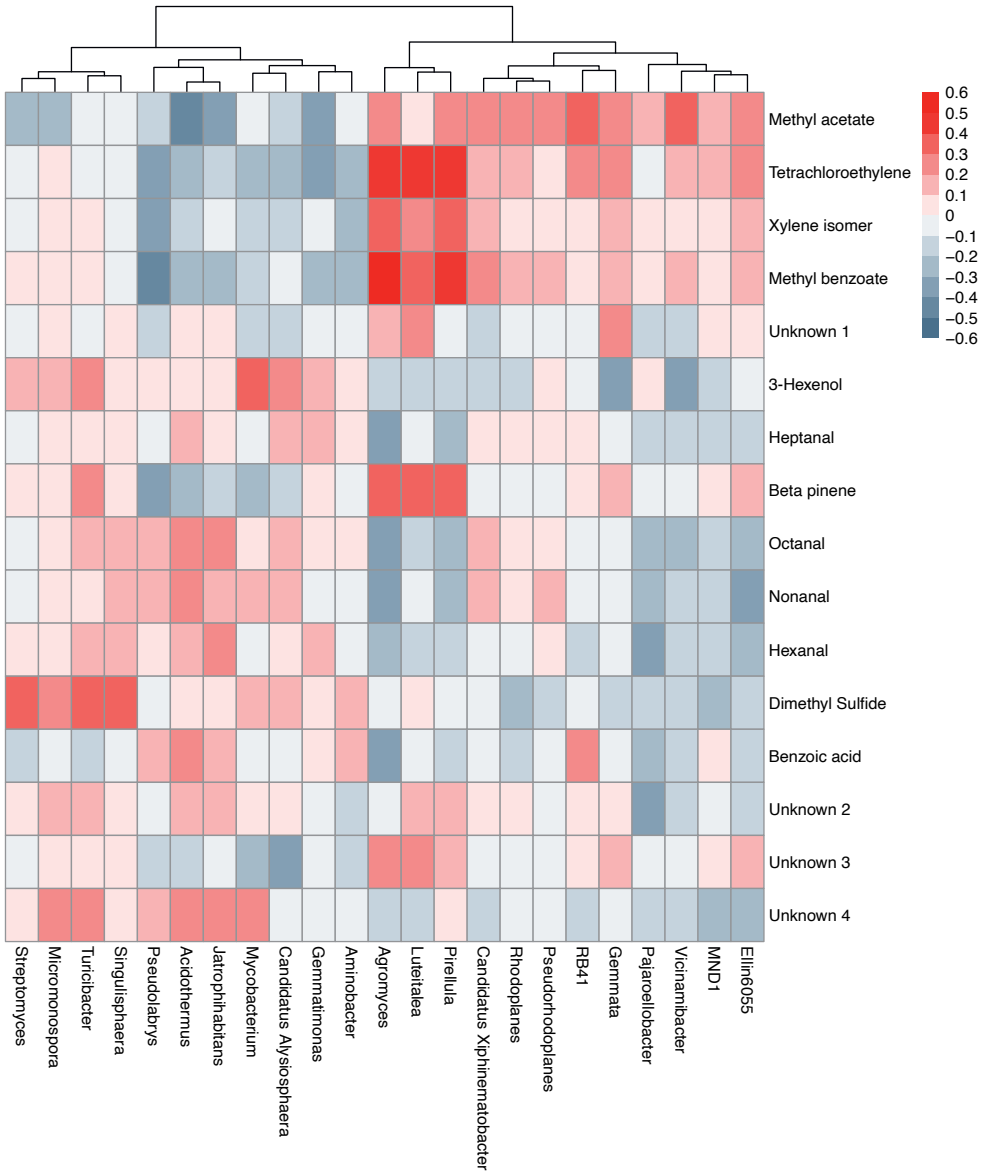


Figure S4.6: Heatmap created from correlation matrix (Spearman) from indicative mVOCs and indicative bacterial genera, identified with TITAN analysis

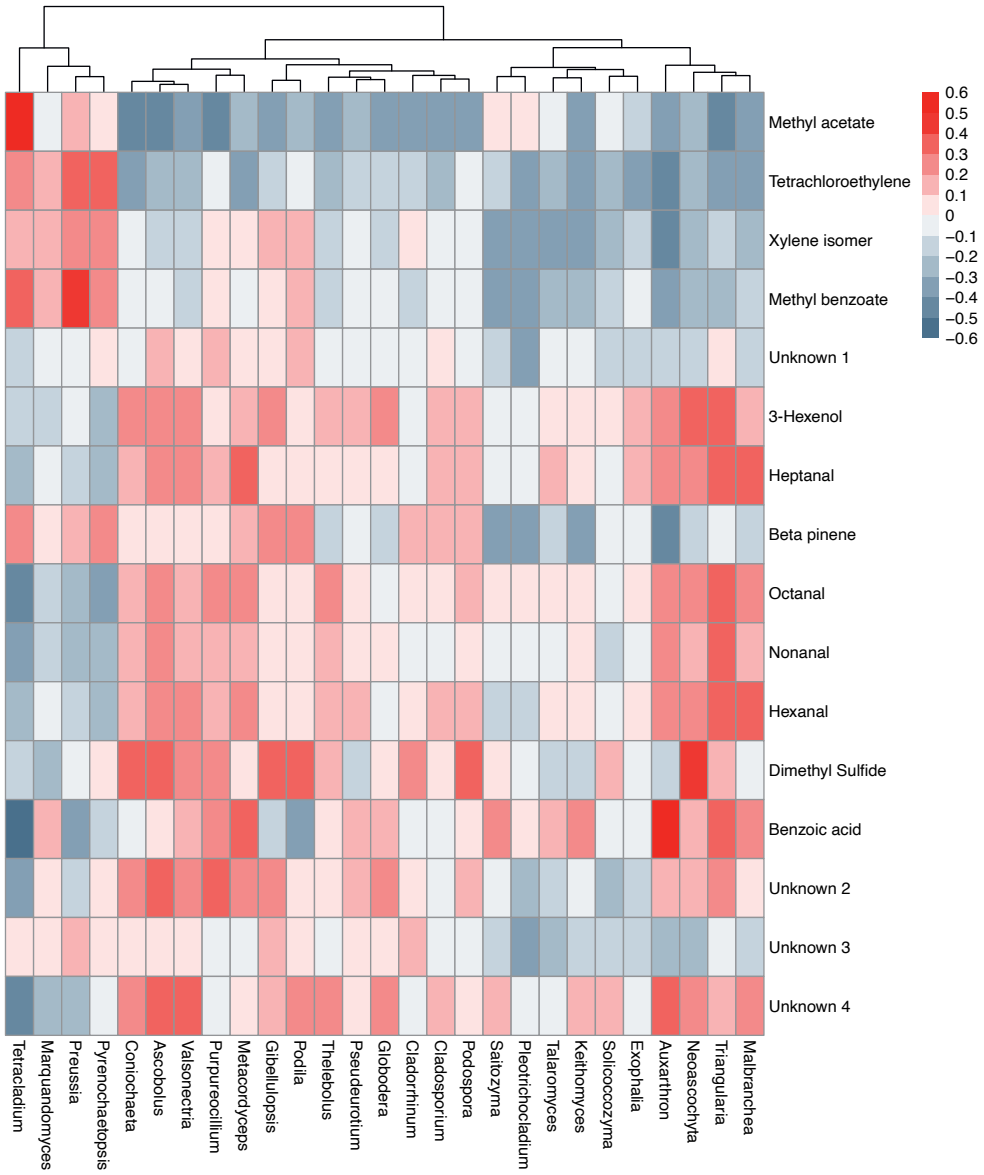


Figure S4.7: Heatmap created from correlation matrix (Spearman) from indicative mVOCs and indicative fungal genera, identified with TITAN analysis.

4.B Supplementary tables

Table S4.1: Questionnaires from landowners, averaged per land use intensity type.

Type	Age grassland (year)	Age management (year)	Grass-cuttings (nr/year)	Grazing (months/year)	Livestock (unit/ha)	Usage of chemical fertilizer	Amount of solid manure (m^3/ha)	Usage of pesticides	Plant community type
Conventional	86	39	3	8	3	Yes	44	Yes	Perennial rye grass
Extensive	56	21	1.5	6.8	2.3	No	17	No	Herb-rich swards
Semi-natural	81	17	0.5	0.0	1.0	No	0	No	Nature inclusive swards

Table S4.2: Results of ANOVA for difference between chemical Volatile Organic Compound (VOC) classes per land use intensity type. Significant differences ($p < 0.05$) between classes are indicated with an asterisk (*).

VOC class	F-value	p-value	Adjusted p-value		
			Conventional – Extensive	Conventional – Semi-natural	Extensive – Semi-natural
Alcohols	0.821	0.446	0.571	0.468	0.984
Aldehydes	4.236	0.020*	0.084	0.022*	0.833
Alkanes	5.710	0.006*	0.027*	0.895	0.008*
Alkenes	0.767	0.682	0.808	0.595	1.000
Amides	1.093	0.343	0.383	0.993	0.447
Aromatic hydrocarbons	0.585	0.561	0.631	0.999	0.610
Carboxylic acids	8.760	0.013*	0.005*	0.108	0.385
Esters	3.787	0.029*	0.736	0.144	0.027*
Ethers	4.634	0.099	0.179	0.058	0.917
Halogenated compounds	12.990	0.002*	0.636	0.001*	0.012*
Hydrocarbons	13.050	0.001*	0.253	0.041*	0.001*
Ketones	0.521	0.771	0.757	0.851	1.000
Monoterpenes	4.430	0.109	0.159	1.000	0.072
Sesquiterpenes	6.628	0.036*	1.000	0.026*	0.063
Sulfur compounds	5.435	0.007*	0.023*	0.013*	0.970
Unknown compounds	2.517	0.091	0.099	0.922	0.207

Table S4.3: Results of pairwise PERMANOVA testing differences in VOC profiles between management intensity levels, based on a Euclidean distance matrix. Each row represents one pairwise comparison between two groups (Semi-natural, Conventional, and Extensive grasslands). Reported measure include the dissimilarity measure used (Bray-Curtis). Benjamini-Hochberg was used for adjusted p-value (p.adjusted). Significance is indicated as: * $p < 0.05$; ns = not significant.

Groups	Df	SumsOfSqs	F.Model	R^2	p.value	p.adjusted	Significance
Semi-natural vs Conventional	1	0.03	1.02	0.03	0.32	0.96	ns
Semi-natural vs Extensive	1	0.12	2.94	0.08	0.05	0.15	ns
Conventional vs Extensive	1	0.13	3.40	0.09	0.03	0.09	ns

Table S4.4: Results of pairwise PERMANOVA testing differences in bacterial community composition (16S rRNA, class level) between management intensity levels, based on Bray-Curtis dissimilarity. Each row represents one pairwise comparison between two groups (Semi-natural, Conventional, and Extensive grasslands). Reported measure include the dissimilarity measure used (Bray-Curtis). Benjamini-Hochberg was used for adjusted p-value (p.adjusted). Significance is indicated as: * $p < 0.05$; ns = not significant.

Groups	Measure	F	R^2	p.value	p.adjusted	Significance
Semi-natural vs Conventional	bray	2.17	0.06	0.04	0.06	ns
Semi-natural vs Extensive	bray	2.15	0.06	0.04	0.06	ns
Conventional vs Extensive	bray	1.87	0.05	0.09	0.09	ns

Table S4.5: Results of pairwise PERMANOVA testing differences in fungal community composition (ITS2, phylum level) between management intensity levels, based on Bray-Curtis dissimilarity. Each row represents one pairwise comparison between two groups (Semi-natural, Conventional, and Extensive grasslands). Reported measure include the dissimilarity measure used (Bray-Curtis). Benjamini-Hochberg was used for adjusted p-value (p.adjusted). Significance is indicated as: *** $p < 0.001$; ns = not significant.

Groups	Measure	F	R^2	p.value	p.adjusted	Significance
Semi-natural vs Conventional	bray	8.59	0.20	0.001	0.001	***
Semi-natural vs Extensive	bray	5.01	0.13	0.001	0.001	***
Conventional vs Extensive	bray	3.68	0.10	0.001	0.001	***

Table S4.6: Spearman correlation coefficients (Correlation) between soil abiotic variables and bacterial (16S, class level) and fungal (ITS2, phylum level) community compositions, represented by the first axis extracted from a Principal Coordinates Analysis (PCoA) based on Bray-Curtis dissimilarity. Significance is indicated as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns = not significant.

	Bacterial community composition			Fungal community composition		
Variable	**Correlation**	**p.value**	**Significance**	**Correlation**	**p.value**	**Significance**
Moisture content (%)	0.14	0.29	ns	0.41	0.00	**
Soil bulk density (0–10 cm)	-0.12	0.40	ns	-0.11	0.44	ns
Organic matter content (%)	0.13	0.36	ns	0.25	0.07	ns
Nitrogen (%)	0.06	0.68	ns	0.10	0.49	ns
Soil organic carbon (%)	0.13	0.34	ns	0.40	0.00	**
pH	0.17	0.22	ns	0.53	0.00	***
Al (kg/ha)	-0.13	0.34	ns	-0.28	0.04	*
Ca (kg/ha)	0.09	0.49	ns	0.27	0.05	ns
Fe (kg/ha)	-0.05	0.71	ns	-0.21	0.13	ns
K (kg/ha)	0.17	0.21	ns	-0.15	0.28	ns
Mg (kg/ha)	-0.24	0.08	ns	-0.50	0.00	***
Mn (kg/ha)	-0.31	0.02	*	-0.47	0.00	***
P (kg/ha)	0.04	0.77	ns	-0.36	0.01	**
S (kg/ha)	0.04	0.76	ns	-0.37	0.01	**
Olsen P (kg/ha)				0.00	0.99	ns
NO_3^- (kg/ha)	0.02	0.88	ns	0.00	0.98	ns
NH_4^+ (kg/ha)	-0.10	0.49	ns	-0.29	0.03	*

Table S4.7: Spearman correlation coefficients (Correlation) between the management intensity gradient and soil abiotic variables. The management gradient represents an ordinal ranking from Conventional (most intensive) to Extensive to Semi-natural (least intensive). Significance is indicated as: * p < 0.05, ** p < 0.01, *** p < 0.001; ns = not significant.

Variable	Correlation	Significance
Moisture content (%)	0.38	**
Soil bulk density (0–10 cm)	-0.23	ns
Organic matter content (%)	0.38	**
Nitrogen (%)	0.17	ns
Soil organic carbon (%)	0.49	***
pH	0.34	*
Al (kg/ha)	-0.20	ns
Ca (kg/ha)	0.34	*
Fe (kg/ha)	-0.31	*
K (kg/ha)	-0.38	**
Mg (kg/ha)	-0.41	**
Mn (kg/ha)	-0.39	**
P (kg/ha)	-0.56	***
S (kg/ha)	-0.48	***
Olsen P (kg/ha)	-0.15	ns
NO_3^- (kg/ha)	0.14	ns
NH_4^+ (kg/ha)	-0.23	ns

Table S4.8: List of indicative mVOCs identified with TITAN2 analyses. Related bacterial and fungal genera from mVOC library (3.0) are recorded in the second column. The genera that are overlapping with the bacterial and fungal TITAN analysis are underlined.

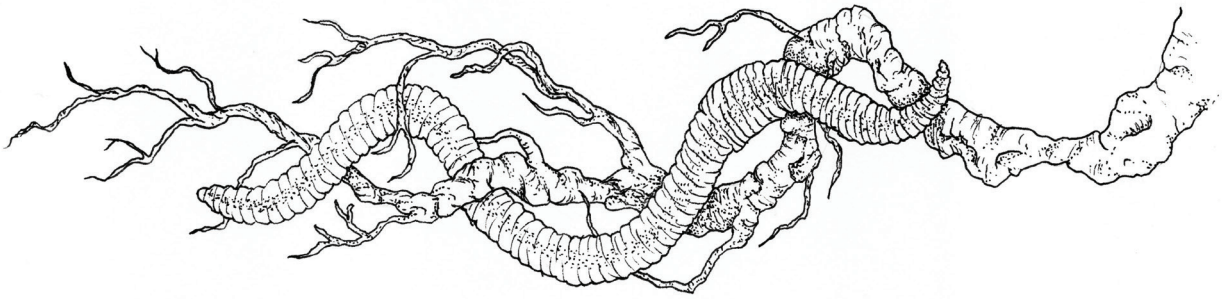
mVOC	mVOC library
Heptanal	Bacteria; Lactobacillus Rhamnosus. Fungi; Fusarium Graminearum, Pleurotus, Trichodema, Tuber, Streptococcus, Fomitopsis, Ganoderma, Trametes
Hexanal	Bacteria; Carnobacterium, Lactobacillus, Pseudomonas, Serratia. Fungi; Aspergillus, Fusarium, Muscodor, Pennicilium, Pleurotus, Rhizopus, Trichodema, Tuber
Octanal	Fungi; Aspergillus
Nonanal	Bacteria; Bacillus, Calothrix, Carnobacterium, Chondromyces, Lactobacillus, Paenibacillus, Pedobacter, Phormidium, Plectonema, Pseudomonas, Rivularia, Serratia, Tolypothrix. Fungi; Fusarium, Penicillium, Pleurotus, Trichodema, Tuber
Dimethyl sulfide	Bacteria; Burkholderia, Alcaligenes, Alpha Proteobacteria, Clodistrium, Collimonas, Desulfovibrio, Gamma proteobacteria, Enterobacter, Lactobacillus, Lactococcus, Paenbacillus, Parasporobacterium, Pedobacter, Pseudomonas, Pseudonocardia, Ralstonia, Saccharomonospora, Serratia, Mycobacterium. Fungi; Chaetomium, Clostridium, Eurotium, Fusarium, Penicillium, Tuber
Hexenol-3	Aspergillus, Penicllium, Rhizopus
Benzoic acid	Bacteria; Bacillus. Fungi; Agaricus, Bjerkandera, Phellinus, Sparassis
Beta-pinene	Bacteria; Collimonas, Streptomyces, Paenibacillus, Pedobacter, Serratia, Streptococcus. Fungi; Antrodia, Penicillium, Amanita, Gomphidius, Mycena, Suillus, Tricholoma
Xylene isomer	Bacteria; Serratia, Carnobacterium, Pseudomonas. Fungi; Saccharomyces, Tuber, Paecilomyces
Methyl benzoate	Bacteria; Pseudomonas, Salinispora, Stigmatella, Streptomyces. Fungi; Cladosporium, Phellinus, Phialophora
Tetrachlororethylene	Bacteria; Burkholderia
Methyl acetate	Fungi; Tuber, Paecilomyces, Penicillium



Photograph taken at the author's family farm in Zeeland, the Netherlands.

5

Intermezzo: What would make a useful soil(-life) sensor? Perspectives from farmers and soil scientists



5.1 Introduction

This intermezzo integrates insights from two complementary activities exploring the potential development of soil(-life) sensing technologies. First, a two-year educational collaboration between OnePlanet Research Center, Radboud University, and Aeres Hogeschool Dronten investigated farmers perspectives on soil sensing through interview-based student projects. Second, a scientific workshop on soil sensing technologies was organised during the 2nd Symposium of the National Center for Soil Ecology (NCSE), where soil researchers discussed requirements and challenges for developing volatile organic compound (VOC)–based soil sensors. These activities addressed similar questions from different perspectives and together provide insight into both user needs and scientific feasibility.

5.1.1 Farmer perspectives: monitoring change during incremental transitions

The educational collaboration consisted of two phases. In the first year, two HBO students conducted exploratory interviews with farmers to identify expectations for a soil-life sensor. Their findings suggested that conventional farmers were more interested in monitoring technologies than farmers who had already transitioned toward extensive or biological management. Farmers who had already implemented long-term changes often perceived their soils as functioning well and therefore saw less added value in additional monitoring. In contrast, conventional farmers described management as a process of gradual adjustments and were interested in tools that could reveal whether such changes influenced soil life.

In the second year, an MSc student and HBO students expanded this work with interviews involving additional farmers (24 in total across conventional dairy, conventional arable and biological dairy systems). Although interview protocols differed between years, results were consistent with the earlier exploration: interest in soil sensing was highest among conventional farmers and lowest among biological dairy farmers. Importantly, farmers did not associate sensors primarily with transitions to organic agriculture, but with decision support, faster feedback compared to periodic laboratory analyzes, and improved understanding of soil responses to management. In interviews, farmers emphasized that a soil sensor preferably should help them understand whether soils are changing, identify spatial variability within fields, and provide actionable management guidance. Desired information included early warning signals for problems, causal explanations combined with management recommendations, benchmarking relative to reference values or previous measurements, and spatial differentiation between field

zones. Farmers also frequently mentioned familiar soil fertility indicators, for example pH and soil organic matter, reflecting current reliance on periodic laboratory analyses and a desire for more frequent or continuous measurements. Adoption was considered conditional on practical usability, clear interpretation linked to management decisions, and economic feasibility.

Together, these findings suggest that monitoring tools may be most valuable for farmers who are actively adjusting management practices, highlighting the importance of detecting trajectories of change rather than static soil states.

5.1.2 Scientific perspectives: requirements for VOC-based soil sensing

Complementary insights emerged from the NCSE workshop with soil researchers, which focused specifically on the development of VOC-based soil sensing technologies. Participants emphasised that such sensors should preferably measure process-relevant signals rather than only chemical composition. Minimum requirements included automated and programmable measurement intervals, high-throughput capability, portability for in situ measurements, and the ability to link VOC patterns to ecological processes such as disease suppression, mineralization pathways, organic matter decomposition routes, presence or absence of pathogens, and bioremediation activity. Researchers also highlighted the potential value of sensors capable of identifying microbial community composition through VOC signatures, including detection of pathogens, beneficial organisms, or microbial communities suited for specific land uses. However, several scientific challenges were identified, including limited reference databases, calibration difficulties, soil heterogeneity, accuracy constraints, and the need for specialised analytical expertise and instrumentation.

5.1.3 Integrating user needs and scientific feasibility

Although originating from different positions, farmer and researcher perspectives converged on several points. Both groups emphasised the importance of linking measurements to ecological processes and management interpretation rather than providing raw data alone. At the same time, scientific discussions highlighted technical and methodological challenges that must be overcome before such tools can become operational in agricultural practice. These combined insights illustrate that developing soil sensing technologies requires not only advances in measurement science but also alignment with stakeholder needs, particularly the ability to detect meaningful changes during management transitions. Such alignment may enhance the relevance of monitoring tools for supporting agricultural extensification trajectories.

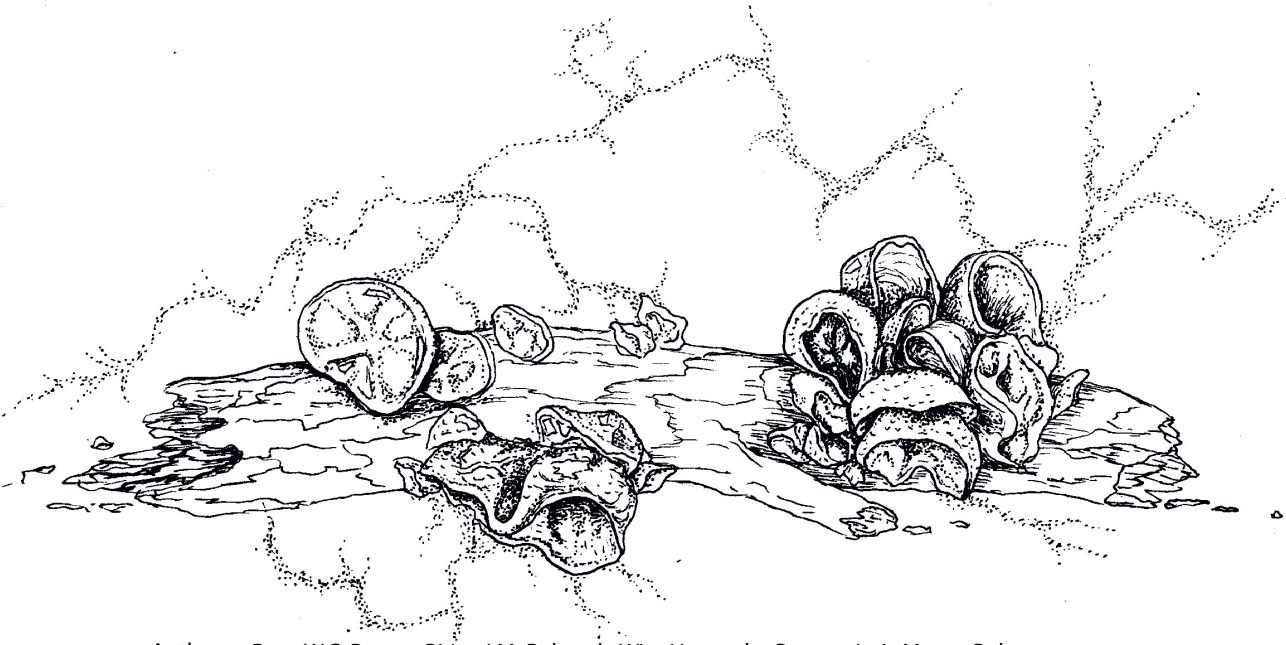


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Photograph taken at the author's family farm in Zeeland, the Netherlands.

6

The potential of microbial Volatile Organic Compounds as a microbial proxy for metabolic functioning in grassland soils



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In preparation

Abstract

Microbial functioning is central to soil health, yet robust and integrative proxies for monitoring it under field conditions remain limited. Microbial volatile organic compounds (mVOCs) have recently emerged as a promising non-destructive fingerprint of soil microbial activity, but their capacity to evaluate consequences of land use for soil functioning has not been evaluated yet. Here, we compare the performance of mVOC profiles with that of extracellular enzyme activities (EEA), which are used as a proxy for soil microbial functioning. We compared both approaches across 18 grassland fields spanning a gradient of land-use intensity from conventional, extensive, to semi-natural). We assessed (i) whether the unsupervised multivariate structure of each proxy aligns with post-priori land-use types, (ii) if the two proxies covary across sites, and (iii) how their supervised classification performance compares. Results showed that in unsupervised ordinations neither proxy produced clear separation among land-use types, and that the multivariate structures of the two proxies did not correspond. Therefore the results suggested that mVOCs and enzymes capture distinct biological signals and that soil functioning does not map neatly onto discrete management labels. Despite this, canonical correlation analysis revealed a strong shared gradient between mVOC and enzyme profiles (r = 0.82), demonstrating meaningful multivariate coupling between the two proxies. Unexpectedly, mVOC profiles outperformed enzyme activities in supervised classification of land-use types (balanced accuracy 0.95 vs. 0.81), with dimethyl sulfide and methyl acetate emerging as the most discriminating compounds for conventional land use. These results suggest that mVOCs capture emergent metabolic properties of microbial communities not fully represented by enzyme assays, and that the two proxies may be considered complementary rather than competing indicators. We discuss implications for multi-proxy soil monitoring and the broader challenge of establishing reference values that provide a benchmark for soil functioning.

6.1 Introduction

GLOBALLY, land use intensification poses one of the biggest threats to ecosystems and the services they provide to humans, including food and fiber production, climate regulation and the provision of clean air and water (Allan et al., 2015; IPBES, 2019). Soils and soil biodiversity contribute to almost all ecosystem services (Delgado-Baquerizo et al., 2025), however, they remain poorly monitored and understood (Bardgett et al., 2023). Therefore, the European Parliament and Council have formally adopted the Soil Monitoring Directive, establishing the first-ever EU-wide framework for assessing and monitoring soils. The directive recognizes that soil biological diversity underpins ecosystem functioning and forms the basis of healthy soils that support ecosystem services on which human societies depend, while being threatened increasing land-use intensity (Council of the EU, 2025). To monitor soil biodiversity and functioning effectively, there is a lack of robust, integrative and easily measurable proxies capable of capturing how external pressures alter soil communities and their activity (Fierer, Wood & Bueno de Mesquita, 2021; Bünemann et al., 2018). During the past two decades, advances in soil research have begun to open the "black box" of soil, revealing its biology, chemistry and physics. Microbes and invertebrates are key drivers of nutrient cycling, organic matter turnover, and soil structure formation, and thus central to the concept of soil health (Bardgett & Putten, 2014; Creamer et al., 2022). However, physiological and metabolic understanding of how complex soil communities acquire nutrients, modify their environment, and influence soil processes remains incomplete (Briones, 2014).

While biological indicators based on molecular sequencing in the lab of field-collected soil samples mostly reflect structural and functional community composition (Fierer et al., 2021), integrative fingerprints that reflect both community composition and activity in situ are still rare (Bünemann et al., 2018; Lehman et al., 2015). Many functional assessments are carried out under controlled laboratory conditions, which is not representative to conditions in the field (Burns et al., 2013). What is missing are innovative approaches that link microbial presence, abundance, and metabolic activity in a more integrative and time-dynamic manner that can applied directly in the field. Recent advances suggest that microbial volatile organic compounds (mVOCs) may be helpful to enhance integration of activities of soil biota over time (Brown et al. 2021; Boone et al., 2025; Insam & Seewald, 2010). These secondary metabolites provide soils with their characteristic smell and are produced by both plants and soil-borne microbes (bacteria, fungi, archaea and protists), although the production by archaea and protists remains largely understudied (Yang et al., 2024; Weisskopf, Schulz & Garbeva, 2021; Tang, Schurgers & Rinnan, 2019).

Other soil eukaryotes, such as nematodes, micro-arthropods and earthworms, may in-

directly contribute to the volatiles released from soils by feeding on plant roots and microbes (Zaroubi et al., 2022; Becher et al., 2020). The soil-borne volatiles originate from a broad spectrum of microbial metabolic processes, influenced by the diversity, abundance and activity of the microbial community (Abis et al., 2018; Bastos & Magan, 2007; McNeal & Herbert, 2009). Studies on the change in mVOC profiles through influencing the microbial community have been measured mostly under controlled circumstances (Brown et al., 2021; Abis et al., 2018). Nevertheless, recent research has shown that volatiles may also be captured under field conditions (Wester-Larsen et al., 2020; Boone et al., 2025). Specifically, Boone et al., (2025) identified 16 VOC compounds that systematically varied in relative abundances with intensity of management and relative abundances of specific microbial taxa. Because mVOCs integrate information about community composition, abundance, and metabolic state (Boone et al., 2025), mVOC profiles have the potential to serve as non-destructive, dynamic fingerprints of the microbial community. However the representativeness of volatiles first needs to be validated under field conditions (Brown et al., 2021).

Traditionally, soil microbial functioning has been assessed using proxies such as substrate induced respiration and extracellular enzyme activity (EEA), which targets specific biochemical pathways under standardised laboratory conditions (Burns et al., 2013). EEA has been widely used to evaluate the effects of land-use intensity on microbial communities (Creamer et al., 2009; Pérez-Guzmán et al., 2021; Zhou, Wang, & Luo, 2020) and is recognised as a key biological indicator (Stott, 2019; Bünemann et al., 2018). Because microbes secrete enzymes to degrade organic matter and release nutrients, enzyme activity is often tightly associated with organic matter content, microbial biomass, and general activity (Winding, Hund-Rinke & Rutgers, 2005; Nannipieri, Trasar-Cepeda & Dick, 2018). Changes in enzyme activities can reveal microbial responses to disturbance or management practices even before shifts in community structure become apparent (Dick., 1994; Lehman et al., 2015). Despite these advantages, enzyme assays remain targeted and context-dependent, and their results are sensitive to soil properties such as pH, temperature, and moisture (Nannipieri, Trasar-Cepeda & Dick, 2018).

Compared to measuring potential enzymatic activities, mVOC analysis provides an untargeted volatile profile that may reflect a broader spectrum of microbial processes (including those driven by other soil organisms) directly in situ. Importantly, these indicators target different levels of microbial functioning: enzyme assays quantify potential hydrolytic capacity under controlled conditions, whereas mVOCs capture metabolic processes in situ. Although enzyme assays require destructive sampling and incubation, mVOCs can be captured non-destructively from soil airspace and analysed using gas chromatography–mass spectrometry (GC–MS) without extensive post-processing (Insam

& Seewald, 2010). Rather than replacing established indicators, mVOCs could thus offer a complementary dimension, linking microbial identity with real-time metabolic activity and expanding the toolbox for soil biodiversity and function monitoring.

Land use intensity can be conceptualised either as a continuous gradient, reflecting gradual variation in inputs and disturbance (Armengot et al., 2011), or as discrete land-use types that group fields based on shared management regimes (e.g. conventional vs organic). Gradient-based approaches are particularly useful for identifying threshold responses and continuous shifts in microbial communities and processes, whereas type-based approaches are commonly applied in soil monitoring and management contexts, where categorical distinctions are required for interpretation and decision-making. While mVOC indicators have recently been identified along a continuous management intensity gradient (Boone et al., 2025), it remains unclear whether these same compounds may be used to reliably discriminate land-use categories. Bridging these two perspectives is a necessary step if mVOCs are to be applied as practical soil health indicators.

Here, we test whether the 16 mVOCs that have been identified along a continuous management gradient (Boone et al., 2025) can also distinguish among their corresponding discrete groups; This would be a necessary step in assessing if mVOCs may serve as practical classification tools. In addition, we compare the performance of mVOC profiles with that in the enzyme activities, which is a well-established microbial proxy traditionally used to classify land-use intensity.

Based on the established use of extracellular enzyme activity as a proxy for land-use intensity, we hypothesised that enzyme activity profiles would show a strong correspondence with post-priori land-use types. We further hypothesized that the mVOCs profiles would also reflect land-use types, but less clearly than enzyme activities, as mVOCs integrate a broader range of microbial metabolic processes that are not exclusively driven by land-use intensity. Second, we hypothesised that mVOC and enzyme profiles would covariate across sites, reflecting their shared sensitivity to land-use–driven changes in soil microbial communities and functioning. Third, we hypothesised that enzyme activities would outperform mVOC profiles in supervised classification of land-use types, owing to the more targeted and mechanistically established links between specific enzymes and land-use intensity, compared with the more integrative and novel nature of mVOCs. These hypotheses correspond to three analytical questions: (i) whether the unsupervised multivariate structure of mVOC and enzyme profiles aligns with post-priori land-use types, (ii) whether both proxies covary across sites, and (iii) how their predictive performance compares when used for supervised classification of land-use types.

6.2 Materials and methods

6.2.1 Post-priori management labels

This study was conducted in the Ooijpolder region near Nijmegen, the Netherlands (51°51′55.6″N, 5°54′36.5″E). This region has a long agricultural history and exhibits wide variation in grassland management. In September 2022, 18 grasslands were selected in collaboration with farmers and landowners. Each field had been managed consistently for at least 10 years. Management information was collected through farmer surveys, including fertilisation, pesticide use, grazing, and mowing practices, and plant species richness was quantified via field-based surveys. Based on these management data, grasslands were post-priori categorized (see Boone et al., 2025) into three land-use intensity types:

1. **Conventional**: Perennial ryegrass (Lolium perenne L.) monocultures receiving chemical inputs (fertilizers and pesticides) (n=6);
2. **Extensive**: Herb-rich swards with no pesticides and only organic manure applications (n=4);
3. **Semi-natural**: Non-productive, herb-rich grasslands with no manure inputs (n=6).

6.2.2 Proxies

This study integrates two previously collected datasets derived from the same soil samples: microbial volatile organic compound (mVOC) data described in Boone et al., (2025), and extracellular enzymatic activity data described in Chapter 3. Both datasets were generated from soils collected from the same fields and sampling campaign in 2022, enabling direct comparison between proxy types.

6.2.3 Microbial Volatile Organic Compounds (mVOCs)

mVOCs were extracted from soil samples at the same time as soil collection (see Boone et al., 2025) for detailed protocols). Boone et al., (2025) identified 16 mVOC compounds that served as indicators of land-use intensity thresholds using Threshold Indicator Taxa Analysis (TITAN) (Baker and King., 2010). These included heptanal, hexanal, octanal, nonanal, dimethyl sulfide, 3-hexenol, benzoic acid, β-pinene, an isomer of xylene, methyl benzoate, tetrachloroethylene, methyl acetate, and four unidentified compounds.

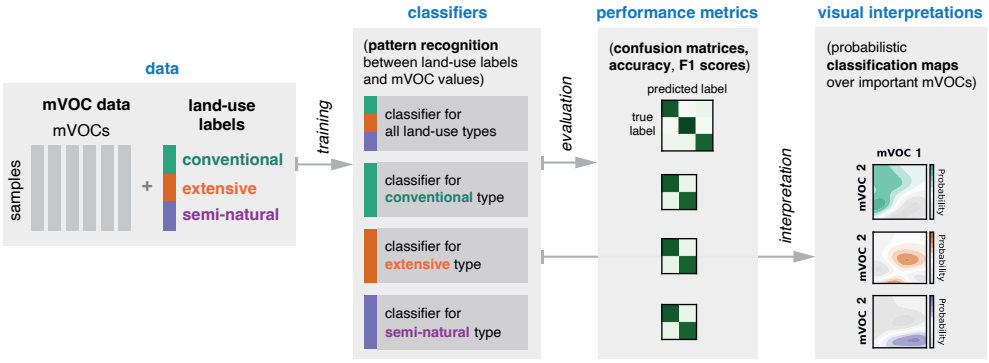


Figure 6.1: Overview of the classification step of the data analysis

In that study, the mVOCs were identified along a gradient of management intensity derived from farmer survey data, rather than discrete management categories. In the present analysis, we re-evaluate the same dataset along the post-priori management clusters, allowing comparison with enzyme data that were originally selected for their ability to discriminate between land-use types, rather than continuous gradients. The dataset contains the normalised peak areas of the 16 indicative mVOCs identified by the TITAN analysis.

6.2.4 Enzymatic activity assays

For the comparison of mVOCs with a traditional proxy for soil health, we used Enzymatic Activity (EA) assays. For our study, we used extracellular enzymatic activity data from nine enzymes (Chapter 3) that are relevant for detecting land-use intensity effects on soil function (Creamer et al., 2009; Zhou, Wang, & Luo 2020): β-xylosidase (XYLO), sulfatase (SULF), acid phosphatase (PHOS), N-acetyl-β-glucosaminidase (NAG), L-leucine aminopeptidase (Leucine), β-glucosidase (BG), β-galactosaminidase (Galamini), β-galactosidase (Galadase), and β-cellobiohydrolase (Cello).

6.2.5 Data analysis

All variables (mVOCs and enzymatic activity assays) were standardized to zero mean and unit variance (z-score normalization) prior to analysis.

Unsupervised ordination

We first explored the multivariate structure of the 16 mVOCs and 9 enzymatic activities using unconstrained, unsupervised dimensionality reduction (Principal Component Anal-

ysis, PCA). Ordinations were computed without using any land-use type information and used for exploratory visualization only. Known land-use types were subsequently overlaid post hoc to assess whether the dominant multivariate structure aligned with post-priori land-use classification.

Covariation

The Spearman correlation was used to calculate pair-wise correlations between mVOCs and Enzymes and visualized on a heat map, showing only significant correlations ($p < 0.05$). Groups of enzymes and mVOCs with similar correlation patterns were obtained with a bipartite clustering algorithm (Platig et al., 2016). The groups of this bipartite analysis are visualized with dashed lines.

To assess the coordinated variation between the two proxy types beyond pairwise correlations, we applied canonical correlation analysis (CCA) (Wegelin et al., 2000). CCA identifies linear combinations (canonical variates) of mVOCs and enzymes that maximize the correlation between the two datasets, thus revealing latent gradients shared between both proxies.

For each canonical axis, CCA combines the mVOCs and enzymes into weighted composite scores per sample, the weights chosen to maximise the correlation between the two datasets. We then calculated these paired scores for all 54 samples and quantified how strongly they covaried. The first canonical axes were visualised using scatter plots of these scores. Correlations between the paired scores were then evaluated across the 54 samples. Coefficients for each mVOC and enzyme are shown; these represent the weights used to calculate the composite scores. Variation among samples along each canonical axis arises from differences in their measured mVOC and enzyme values, which are combined using the same set of coefficients.

Classification of land-use types

Based on the overall workflow (Fig. 6.1), after data and label collection, a supervised training process obtained statistical classifiers that recognize and describe the statistical patterns between land use labels and mVOC data. Then a set of performance metrics quantify how well land-use types could be derived from these patterns. Finally, we interpret the classifiers visually, by showing the patterns in the form of two-dimensional probabilistic classification maps over pairs of important mVOCs. Given that EEA and mVOCs represent different functional layers, our comparison focuses on their relative ability to classify land-use types, not on functional equivalence.

VOC selection

For classification, we started with datasets of mVOC profiles (16 key mVOCs; Boone et al., 2025) and enzymatic activity (9 measured enzymes). To ensure a fair comparison between classifiers trained on the two datasets, we equalized the number of predictor variables. For this, we selected the nine most informative mVOCs using a univariate feature selection step that maximized the mutual information between each mVOC and the categorical management label (Ross., 2014).

Classification method

Machine-learning classification. Machine-learning classifiers were trained to recognize land-use types based on the post-priori management labels. Because the type of extensive land use contained only four fields, we used all available data for training, without reserving a separate test set. As a result, our models provide descriptive rather than predictive classifications: they reveal the statistical relationships between the dependent variables (land-use labels) and the independent variables (mVOC profiles or enzyme activities) across the dataset. Classification performance was considered successful when the trained model discriminated among land-use types more effectively than a null model randomly assigning samples to types.

The choice of the statistical model is crucial to avoid overfitting land-use types to individual fields or samples. This requires a low model complexity with low variance and high bias, or smooth and simple decision boundaries between types (Hastie et al., 2009). For our classification approach, we used Support Vector Classifier (SVC) (Hastie et al., 2009), in its scikit-learn Python implementation version 1.4 (Pedregosa et al., 2011). When configured with a nonlinear kernel, the SVC is a nonlinear model, which simplifies the decision boundary between types due to regularisation, but allows any shape of the decision boundary, as dictated by the data. The decision boundaries of low-variance classifiers should yield smooth visualisations for stakeholders, such that the classification process is interpretable. Alternatives such as decision-tree classifiers learn hard thresholds on the independent variables, and thus yield less natural and more fragmented decision boundaries when visualised.

Parameterisation The SVC (Support Vector Classifier) creates a boundary that optimally separates different groups in our data, even when the groups partially overlap. We used a range of settings to support our SVC. Kernel type: we used a radial basis function (RBF) kernel, which allows the model to draw curved boundary lines instead of just straight lines. This helps it handle more complex patterns in the data. Regularization Parameter

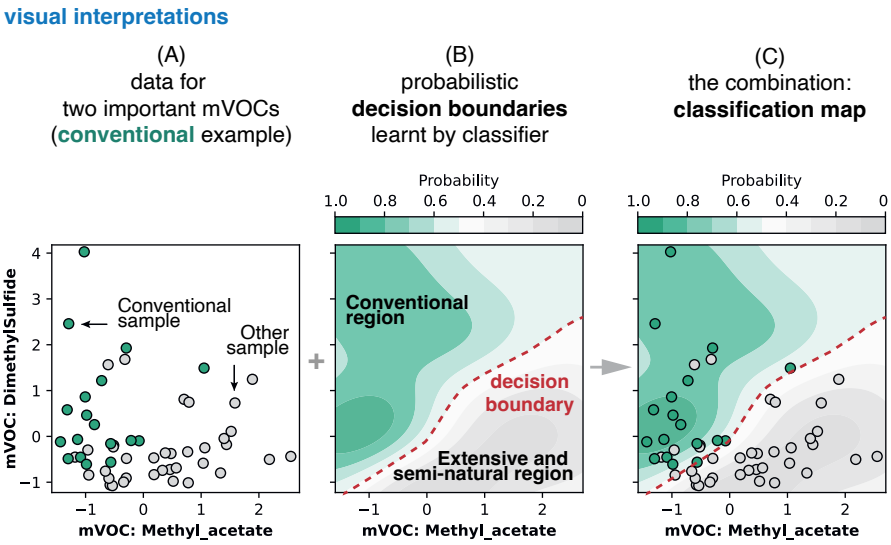


Figure 6.2: Classification map. A visual interpretation of a classifier is composed of (A) a scatterplot of (scaled) mVOC data, and (B) a gradient plot of the probabilistic decision boundaries of the SVC. The combination (C) superimposes the two, such that not only the global patterns between mVOC values and land-use types are shown, but also the minority of misclassified samples.

(C): C controls how smooth the boundary line is. A smaller C value makes a smoother boundary. We tested different values and chose C = 1 as it produced the best balance between accuracy and smoothness. Type balancing: since our data groups have different numbers of samples, we configured the model to treat all groups as equally important when making decisions. Probability output: we configured the model to show how confident it is about each prediction, not just give a yes/no answer. This helps to understand model uncertainty in classification.

Performance metrics We first tested the performance of the classifier when using all 9 mVOCs or 9 enzymes. Our classification tasks were either three-type (with all three land-use types as labels), or two-type (semi-natural land use versus two others combined, and similarly for the other two land uses). As evaluation metrics for these tasks, we provide confusion matrices, which make clear the recall and precision per type as well as how the errors distribute, as well as the F1 score for the type of interest (a harmonic mean of the precision and recall). For three-type tasks, we add the balanced accuracy (the average of recalls per type), which suits classification tasks with imbalanced types (Pedregosa et al., 2011). All evaluation metrics range in the interval [0,1], with 1 meaning perfect performance. For a null model which guesses the type of a sample randomly, the metrics equal $\frac{1}{\#\text{ types}}$, so 0.5 for a binary task and 0.33 for a three-type task.

Visual interpretations Trained two-type SVC classifiers can be interpreted in their en-

tirety. We provide fine-grained visual interpretations, which allows the patterns between selected mVOCs and land-use types to be understood at a glance. Because visualizations are two-dimensional, we systematically retrain a new two-type SVC classifier on every pair of mVOCs from the nine mVOCs selected. If an mVOC pair shows good classification performance (F1 score above 0.66, compared to a null-model baseline of 0.5), then it can discriminate between types to an extent. Then, we visualise this classifier (as shown in Fig. 6.2). These smaller classifiers trained on only two mVOCs are individually less performant than the classifier trained on all nine mVOCs, so should be used only for a detailed interpretation of the results. Taken together, the visual interpretations of all these smaller classifiers provide an overview of how a land-use classification is made.

6.3 Results

6.3.1 Unsupervised patterns

In contrast to our expectations, unsupervised ordinations of both mVOCs and enzyme activities revealed a multivariate structure that did not resemble post-priori land-use types (Supplementary Fig. S6.3 and S6.4). Ordination also did not show clear separation among land-use types along the first two principal components. Instead, samples from different land-use types largely overlapped in the ordination space and within-field variability was comparable to variability among land-use types. Moreover, ordination from mVOCs and enzyme activities did not visually correspond, indicating limited overlap between the multivariate structures.

6.3.2 Covariation

Partly in accordance to our hypothesis, several mVOCs (including tetrachloroethylene, methyl benzoate, β-pinene, benzoic acid, and the unidentified compound 3) showed strong correlations with specific enzyme activities, whereas others (such as methyl acetate, unknown compound 4, and hexanal) exhibited little or no association (Fig. 6.3). Overall, mVOCs displayed a more consistent pattern of correlations (either positive or negative) across the entire enzyme suite. In contrast, enzymatic activities responded more variably across different mVOCs. Notably, sulfate hydrolase and cellulase activities did not correlate significantly with any mVOC.

Coordinated variation between the two proxy types exists beyond pairwise correlations. Canonical Correlation Analysis (CCA) identified combinations of mVOCs and enzymes that maximise their shared correlation (Fig. 6.4). The first canonical axis revealed that a strong association does exist between certain mVOCs and enzymes, with composite mVOC and

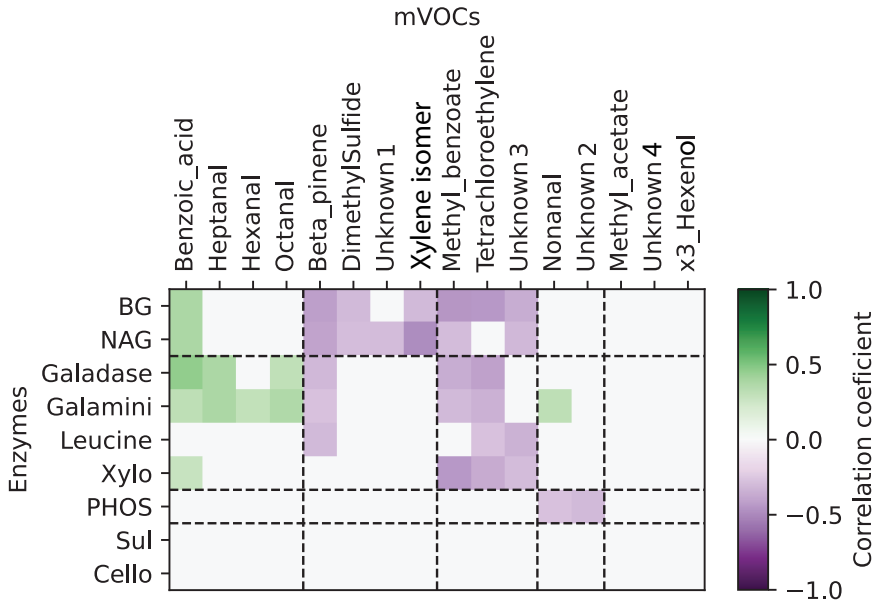


Figure 6.3: Spearman correlation enzymes and mVOCs, only showing significant correlations ($p < 0.05$). Enzymes and mVOCs with similar patterns of correlation are grouped together by bipartite clustering (dashed lines).

enzyme scores highly correlated across the 54 samples ($r = 0.82$). This indicates the presence of a dominant shared gradient linking volatile profiles and extracellular enzyme activities.

The mVOCs and enzymes that contributed positively to this gradient included benzoic acid, heptanal, hexanal, 3-hexenol, octanal, nonanal and unknown compound 4 for mVOCs; sulfatase, L-leucine aminopeptidase, β-galactosidase, β-galactosaminidase and β-glucosidase for enzymes), whereas the negatively weighted variables included β-pinene, dimethyl sulfide, unknown compound 1 and 3, xylene isomer, methyl benzoate for mVOCs). Several variables contributed strongly to shared structure derived from the canonical correlation analysis (Fig. 6.4; Benzoic acid, methyl benzoate, unknown compound 4 for mVOCs; L-Leucine aminopeptidase for enzymes), as reflected by large coefficients, while others showed only minor contributions (tetrachloroethylene, unknown compound 2, methyl acetate for mVOCs; xylosidase, Nacetyl-β-glucosaminidase, acid phosphatase and β-cellobiohydrolase. The second canonical axis (Supplementary Fig. S6.5) explained less shared variation ($r = 0.76$) and represented opposing patterns within each dataset.

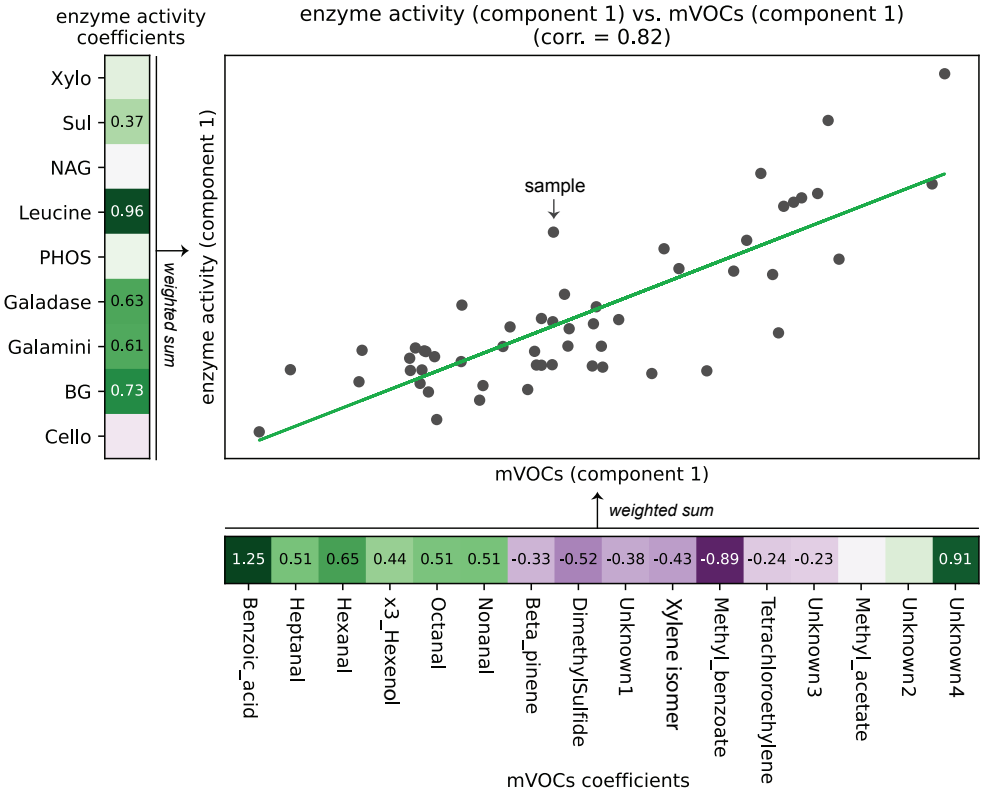


Figure 6.4: Canonical Correlation Analysis (CCA). The figure shows the first canonical axis for soil extracellular enzyme activities and microbial volatile organic compounds (mVOCs). The scatter plot displays paired canonical scores across all 54 samples, with each axis representing a weighted composite of enzyme activities (left heatmap) and mVOCs (bottom heatmap), respectively. The correlation between the two canonical variates was r = 0.82. Heatmap coefficients indicate the weights assigned to each variable in constructing the composite scores; coefficients with small absolute values < 0.2 are omitted for clarity, but all variables were included in the analysis. Positive coefficients (green) indicate variables that increase along the canonical axis; negative coefficients (purple) indicate variables that decrease. Enzyme abbreviations: Xylo = xylosidase, Sul = sulfatase, NAG = N-acetyl-glucosaminidase, PHOS = phosphatase, BG = β-glucosidase, Cello = cellobiohydrolase.

6.3.3 Classification of land-use types

When a three-type classifier was trained using the mVOC profile (a subset of nine selected compounds), it achieved a balanced accuracy of 0.95 (Fig. 6.5), outperforming the model based on all enzyme activities (balanced accuracy: 0.81). This was in contrast to our expectations. When the mVOC dataset was used for pairwise classification between land-use types, the highest accuracy was obtained when distinguishing conventional fields from the other management systems. For the model based on enzyme activity, this was the case for extensive land use. Each of the most important enzymes or mVOC contributed to the

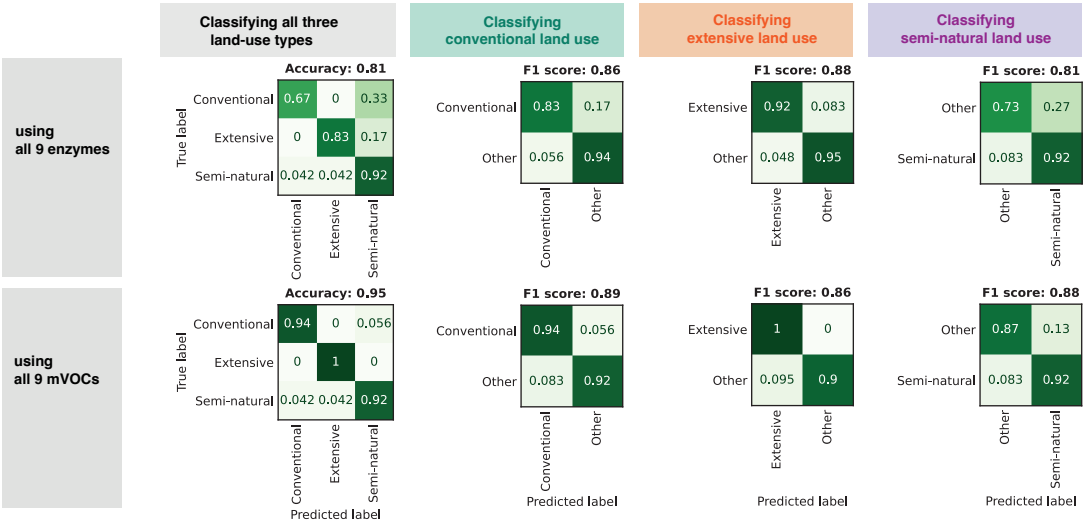


Figure 6.5: Classification performance of Support Vector Classifiers (SVC) trained to discriminate land-use types using either all 9 extracellular enzyme activities (top row) or all 9 mVOCs (bottom row). The leftmost confusion matrices show three-type classification performance (conventional, extensive, and semi-natural land use), with balanced accuracy reported. The remaining three columns show binary classification performance for each land-use type against the other two combined, with F1 scores reported for the type of interest. Cell values represent the proportion of samples per true label assigned to each predicted label (row-normalised); darker green indicates higher proportions. A null model randomly assigning type labels would achieve a balanced accuracy of 0.33 for the three-type task and an F1 score of 0.50 for binary tasks.

separation of one land-use type from the others. We show how the most important pairs of enzymes or mVOC were able to jointly indicate one or more land-use types (we use an F1 score > 0.67 to determine that a pair is important, higher than the null-model F1 score of 0.50 for binary classification tasks).

Overall, mVOC pairs showcased better classification ability compared to enzymes, in contrast to our hypothesis (Fig. S6.1). For the mVOC profiles, four pairs of mVOCs were identified as most effective as proxies for classifying land-use types: dimethylsulfide + methyl acetate (or methyl benzoate, heptanal + dimethylsulfide, dimethylsulfide + benzoic acid and methyl acetate + methyl benzoate. Most pairs were able to discriminate three land-use types from the others, except for methyl acetate + methyl benzoate. The highest accuracy was achieved when combining dimethyl sulfide + methyl acetate (or methyl benzoate) to distinguish conventional land-use type. Overall, the pairs were most effective in distinguishing semi-natural land-use from the other land use types, while extensive land-use types were distinguished the least clearly

For enzymatic activity, similarly, four pairs of enzymes were identified as most effective to distinguish land-use types (Fig. S6.2): Leucine + Galamini (or Galadase, because

the two correlate), Betaglucosidase + Cellosidase, Sulphatase + Galadase and Xylosidase + Galamini (or Galadase). The most effective was combining Leucine + Galamini (or Galadase) to distinguish the semi-natural land use type. The pairs were most effective in distinguishing conventional land-use from the other land-use types, while again extensive land-use was distinguished the least clearly. All four pairs were able to discriminate two land-use types from the others, and specifically, BG + Cello was able to discriminate all three land-use types. The highest F1 score was achieved when combining Sulphatase + Galadase to classify semi-natural land-use types from the others (F1 score: 0.75). In general, semi-natural land-use types was most strongly distinguished from the other types and extensive land-use types the least.

We note that the visual classification maps (both Fig. S6.1 and Fig. S6.2) display relatively simple, but nonlinear decision boundaries. For example, the important pairs of mVOCs (in Fig. S6.1) discriminate the semi-natural land-use type (the third column of classification maps) using almost-circular decision boundaries. It is also clear why the extensive land-use type was the most difficult to discriminate: the decision boundaries for this type (the second column of classification maps) and disconnected into two distinct circular regions, signalling that extensive samples fall into two distinct regimes.

6.4 Discussion

6.4.1 Alignment of unsupervised multivariate structure with land-use

types By comparing an established proxy for soil microbial functioning (extracellular enzyme activities) with a novel, integrative proxy (microbial volatile organic compounds, mVOCs), this study aimed to evaluate not only their mutual alignment and their relative, as well as their complementary power to reflect land use-driven variation in soil microbial functioning. Contrary to our first hypothesis, neither enzyme activity profiles nor mVOC profiles showed clear separation among post-priori land-use types in unsupervised ordinations. For both proxies, samples from different land-use types largely overlapped in multivariate space, and within-field variability was comparable to variability among land-use types. Moreover, the ordination structures derived from enzymes and mVOCs did not visually correspond, indicating limited overlap in the dominant multivariate patterns captured by the two proxies.

Rather than interpreting this as a failure of the proxies themselves, the absence of clear clustering may reflect a more fundamental limitation of post-priori land-use types as indicators of soil functioning. Land-use types are defined by management practices such as fertilisation, pesticide application, and grazing intensity, and while these practices are

known to influence microbial communities and biogeochemical processes, their effects on soil functioning are neither uniform nor synchronous across sites (Geisseler & Scow 2014; Fierer, 2017). The absence of clear clustering therefore does not necessarily imply that either proxy fails to capture meaningful biological variation. Rather, it might suggest that soil functioning itself does not map neatly onto discrete land-use types, at least at the spatial and temporal resolution of this study.

This lack of alignment with land-use types resonates with broader discussions in soil health research, which emphasise that soil functioning is highly context-dependent and not readily reducible to categorical management labels (Bünemann et al., 2018; Fierer, Wood, & Bueno de Mesquita, 2021). This also points to a broader conceptual challenge that will be returned to in the context of monitoring implications: the absence of absolute benchmark reference values against which soil functioning indicators can be interpreted (Schram, Wubs, & van der Putten, 2024). Without such benchmarks, land-use types can only provide limited interpretive guidance, and the observed mismatch between proxies and categories should not be taken as evidence that the proxies are uninformative.

Second, the proxies themselves may not have been sufficiently sensitive to distinguish land-use types. For enzymatic assays specifically, a key limitation is the assumption that measured activity reflects active microbial metabolism. In practice, a considerable proportion of soil enzymatic activity derives from abiontic enzymes; catalytically active proteins stabilised within the soil matrix and no longer associated with living cells (Nannipieri, Trasar-Cepeda & Dick, 2018). Enzymatic assays also measure potential rather than actual activity, conducted under standardised laboratory conditions that may not replicate in-situ dynamics (Burns, 1978; Nannipieri, Trasar-Cepeda & Dick, 2018). Despite these caveats, enzymatic assays are well-established as functional indicators, and the literature provides substantial guidance on how to account for them. For mVOCs, the interpretive framework remains relatively underdeveloped, and less is currently known about suitability of mVOCs as a proxy under field conditions (Brown et al., 2021).

6.4.2 Covariation between mVOCs and enzyme activities

Importantly, the absence of alignment with land-use types does not exclude the two proxies from capturing shared biological variation. As shown (Fig. 6.3 and Fig. 6.4), despite their divergent unsupervised structures, mVOCs and enzyme activities exhibit strong multivariate coupling, indicating that both proxies respond to underlying gradients in soil microbial functioning even when these gradients do not map onto categorical land-use types.

In partial support of our second hypothesis, individual mVOCs and enzyme activities did show significant covariation, despite the lack of correspondence in their overall

ordination structures. Several mVOCs, including tetrachloroethylene, methyl benzoate, β-pinene, and benzoic acid, were significantly correlated with specific enzymes, suggesting links between particular volatile compounds and underlying microbial metabolic pathways, such as organic matter decomposition and aromatic compound turnover. Notably, benzoic acid and methyl benzoate (chemically related benzenoid compounds) emerged as key drivers of the strongest canonical correlation axis. Benzoic acid is produced both by soil microbes and as a plant root exudate, and has been shown to alter microbial community composition by reducing bacterial-to-fungal ratios and promoting specific microbial groups (Liu et al., 2017; Tahir et al., 2017; Gao et al., 2018). Methyl benzoate, the volatile ester derivative of benzoic acid, is widely recognised as a fungal-associated compound, serving as a biomarker for mould growth (Wady & Larsson, 2005) and as one of the dominant VOCs produced by Serendipita spp. that promote plant growth (Venneman et al., 2020).

The large but opposing canonical coefficients of benzoic acid and methyl benzoate indicate that the dominant shared gradient reflects a shift in the relative abundance of these chemically related compounds, rather than their absolute presence. The strong positive loading of L-leucine aminopeptidase on the same axis could suggest that this aromatic shift covaries with enhanced proteolytic activity and organic nitrogen acquisition, consistent with a broader decomposition-related gradient in microbial functioning (Sinsabaugh, Carreiro, & Alvarez., 2002). In contrast, other mVOCs (e.g. methyl acetate, hexanal) showed little or no correlation with the measured enzyme suite.

This suggests that mVOCs capture metabolic activities not represented by standard extracellular enzyme assays, potentially reflecting stress physiology, secondary metabolism, or cross-kingdom interactions such as fungal–bacterial signalling (Tang, Schurgers, & Rinnan, 2019; Weisskopf, Schulz & Garbeva, 2021). Overall, mVOCs displayed more consistent directional correlations (predominantly positive or negative) across enzymes, whereas enzyme responses were more variable across mVOCs. This pattern underscores the context dependence of enzyme activities, which are sensitive to substrate availability and abiotic conditions such as pH, temperature, and moisture (Burns et al., 2013; Nannipieri, Trasar-Cepeda & Dick, 2018). At the same time, canonical correlation analysis revealed a strong multivariate coupling between specific subsets of mVOCs and enzymes, demonstrating that despite heterogeneous pairwise relationships, both proxies capture a shared latent gradient of soil microbial functioning.

6.4.3 Comparative performance in supervised classification

Contrary to our third hypothesis, mVOC profiles outperformed enzyme activities in supervised classification of land-use types, achieving a higher balanced accuracy when all

predictors were used. This suggests that mVOCs may be more effective at discriminating among land-use types than enzyme activities, despite their weaker alignment in unsupervised analyses. One explanation is that enzymes relate more directly to specific biochemical processes, whereas mVOCs integrate responses across multiple metabolic pathways and microbial groups. A study on organically managed tomato fields differing in nutrient management showed that enzymatic activity responded to management, while microbial community composition remained comparatively stable (Bowles et al., 2014). This indicates that enzyme activities can track shifts in resource chemistry or nutrient availability without major restructuring of the microbial community. In contrast, in the dataset analysed here (Chapter 3), fungal community composition responded strongly along the management gradient, whereas enzymatic activity showed limited responsiveness.

Together, these observations suggest that enzyme activities may be more tightly coupled to specific chemical processes, while integrative community-level signals may shift independently, including inter and intra-kingdom interactions (Schulz-Bohm, Martín-Sánchez & Garbeva, 2017). Land-use types, as management-based constructs, may therefore align more closely with integrative metabolic fingerprints than with individual decomposition-related processes. In this sense, mVOCs may better capture emergent properties of microbial communities that arise from combined shifts in composition, abundance, and physiological state (Insam & Seewald, 2010; Peñuelas et al., 2014; Abis et al., 2018). The strongest discrimination achieved by mVOC classifiers occurred between conventional and other land-use types, likely reflecting management-induced changes in substrate inputs, nutrient availability, and disturbance intensity that directly influence microbial metabolism and volatile release patterns (Hartmann & Six, 2023; Insam & Seewald, 2010; Schulz-Bohm, Martín-Sánchez & Garbeva, 2017). By contrast, enzyme activities classified extensive systems more effectively, consistent with their responsiveness to organic matter turnover and C/N cycling rather than rapid management impacts.

The identification of effective mVOC pairs (such as dimethyl sulfide combined with methyl acetate or methyl benzoate, and heptanal combined with dimethyl sulfide) further supports a fingerprint-based interpretation. These combinations likely reflect coordinated shifts in sulfur and carbon cycling under intensification, rather than the action of single compounds (Lomans et al., 2002). Similarly, discriminating enzyme pairs (e.g. leucine aminopeptidase with galactosidase) suggest coordinated regulation of protein and carbohydrate turnover under differing nutrient constraints (Sinsabaugh, Carreiro & Alvarez, 2002; Sinsabaugh et al., 2008). Notably, both proxies struggled to classify extensive systems, which often occupy an intermediate position along land-use gradients. This may reflect greater heterogeneity in management practices within this category (e.g.

variable manure inputs), or increased metabolic stability that reduces contrast with both intensive and semi-natural systems.

6.4.4 Implications for soil monitoring and proxy integration

The slightly higher overall accuracy of mVOC pairs suggests that volatile fingerprints capture emergent metabolic interactions (synergistic or antagonistic processes) not accessible through enzyme assays alone. Because mVOCs represent metabolic output, they integrate effects of community composition, abundance, and physiological state, complementing DNA-based approaches ("who is there") and enzyme assays ("what they can do"). Their non-destructive and field-compatible aspects positions mVOCs as promising candidates for soil monitoring frameworks, including implementation of the EU Soil Monitoring Directive. However, as with other soil biological indicators, there is a need for a benchmark (Schram, Wubs & van der Putten, 2024). In addition, other challenges remain, including compound identification, source attribution (microbial versus plant), and sensitivity to abiotic factors influencing volatilisation. Standardisation of sampling and analytical protocols (e.g. SPME-GC-MS) will be essential to move from proof-of-concept to operational soil-health indicators. Ultimately, the partial overlap but strong multivariate coupling between mVOCs and enzyme activities suggests that these proxies should not be viewed as competing alternatives. Instead, their complementarity points toward multi-proxy frameworks, in which potential process capacity and metabolic activity are jointly assessed to provide a more robust and nuanced evaluation of soil microbial functioning.

6.5 Conclusions

This study compared microbial volatile organic compounds (mVOCs) and extracellular enzyme activities as proxies for soil microbial functioning across a grassland land-use intensity gradient, addressing their alignment with management categories, their mutual covariation, and their relative classification performance. Contrary to expectations, neither proxy produced clear separation among post-priori land-use types in unsupervised ordinations, and the multivariate structures of the two proxies did not correspond. We argue that this reflects not a failure of the proxies, but a fundamental limitation of categorical land-use types as reference points for soil functioning. Soil functioning integrates legacy effects, soil type, vegetation composition, and microclimate in ways that do not resolve neatly into discrete management labels, and the absence of established benchmark reference values further limits the interpretive power of such categories. Despite this, canonical correlation analysis revealed a strong shared gradient between

the two proxies (r = 0.82), confirming that mVOCs and enzyme activities both respond to underlying variation in soil microbial functioning and should be understood as complementary rather than redundant indicators. Enzymes capture potential hydrolytic capacity under standardised conditions, while mVOCs integrate realised metabolic activity in situ, including processes not represented by standard enzyme suites, such as secondary metabolism and cross-kingdom signalling. Contrary to our third hypothesis, mVOC profiles outperformed enzyme activities in supervised classification of land-use types. This suggests that mVOCs capture emergent, community-level metabolic properties that align more closely with the integrative nature of management-driven change than individual decomposition-related processes do. Dimethyl sulfide combined with methyl acetate or methyl benzoate emerged as the most discriminating mVOC pairs, while leucine aminopeptidase combined with galactosidase was most effective among enzyme pairs. Together, these findings position mVOCs as a promising complement to established enzyme-based indicators within multi-proxy soil monitoring frameworks, including those envisioned under the EU Soil Monitoring Directive. Realising this potential will require standardisation of sampling and analytical protocols, improved source attribution of individual compounds, and the development of benchmark reference values against which mVOC-based indicators can be meaningfully interpreted.

6.6 Acknowledgements

We are grateful to numerous colleagues and students who helped with the collection of the previously collected data that was used in this study. In particular, we want to thank students Nienke Sanders, Mila de Bruin, Anouk Wakely, Jan-Willem Takke, Nienke van Delft, Dico Lammers, Willemijn Langedijk. In addition, we want to thank the NCSE for allowing us to host a workshop during the 2nd NCSE symposium to discuss the potential of mVOCs with a group of enthusiastic soil ecology scientists. Finally, we want to thank Gera van Os, Karien Vermeulen and students Jonas Dijkstra, Annelie van Hummel, Douwe van Chastelet, Inge Bos and Loïse Boonman for their contribution to the “soil-life sensor” project.

6.7 Data availability statement

Data available from the Zenodo data repository: https://doi.org/10.5281/zenodo.19451680

Chapter appendix

6.A Supplementary figures

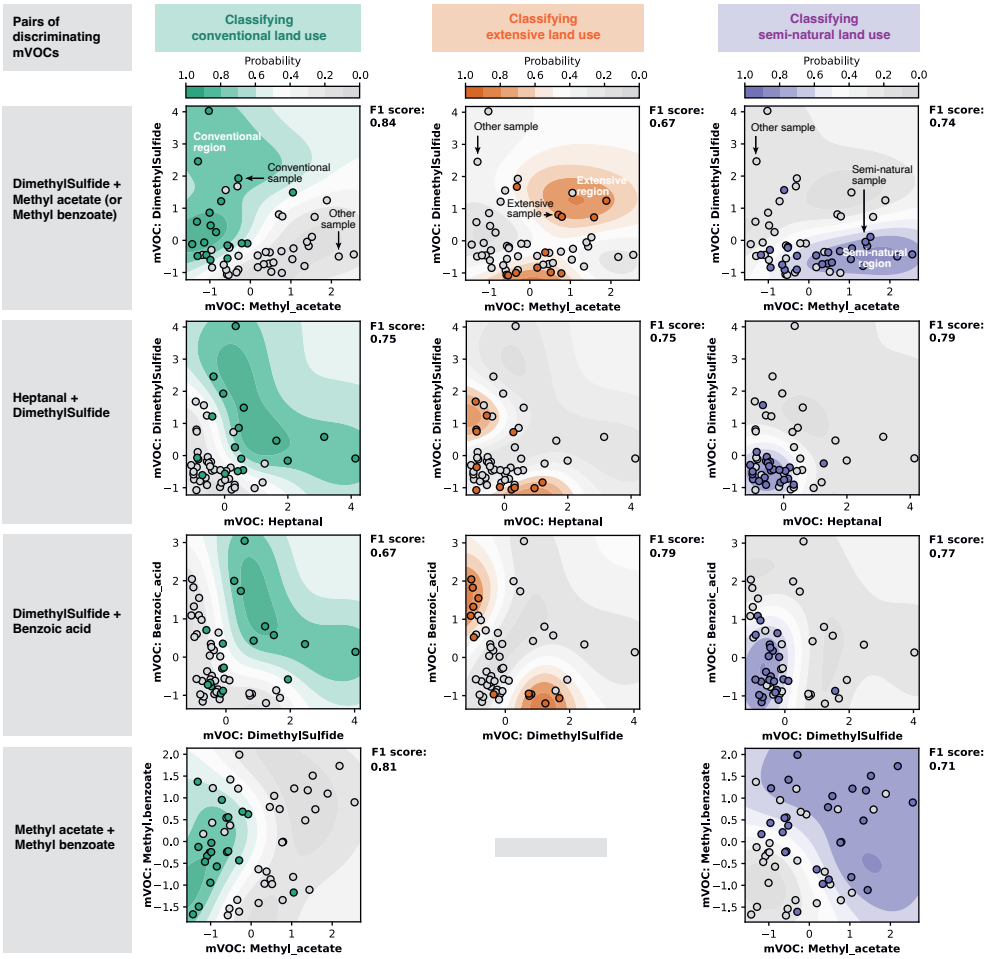


Figure S6.1: Classification of land-use types guided by post-priori land-use labels, using mVOC profiles. Visual probabilistic classification maps for pairs of mVOCs, across land-use types, when the pair can discriminate that land-use label from the others above the accuracy threshold of F1 > 0.67. Each row represents the ability of the pair to distinguish one land-use type (column) for the other land-use types. Axis of each map represents the combination of values for each specific mVOC pairs. The mVOC distributions are scaled (the mean is 0 and the variance is 1). All field samples were used for classification and are coloured by their corresponding post-priori categories are also overlain by the type region. When the sample falls within the wrong type region, the sample classification disagrees with the post-priori label. Sample regions are non-linear (support vector machine using non-linear kernel type).

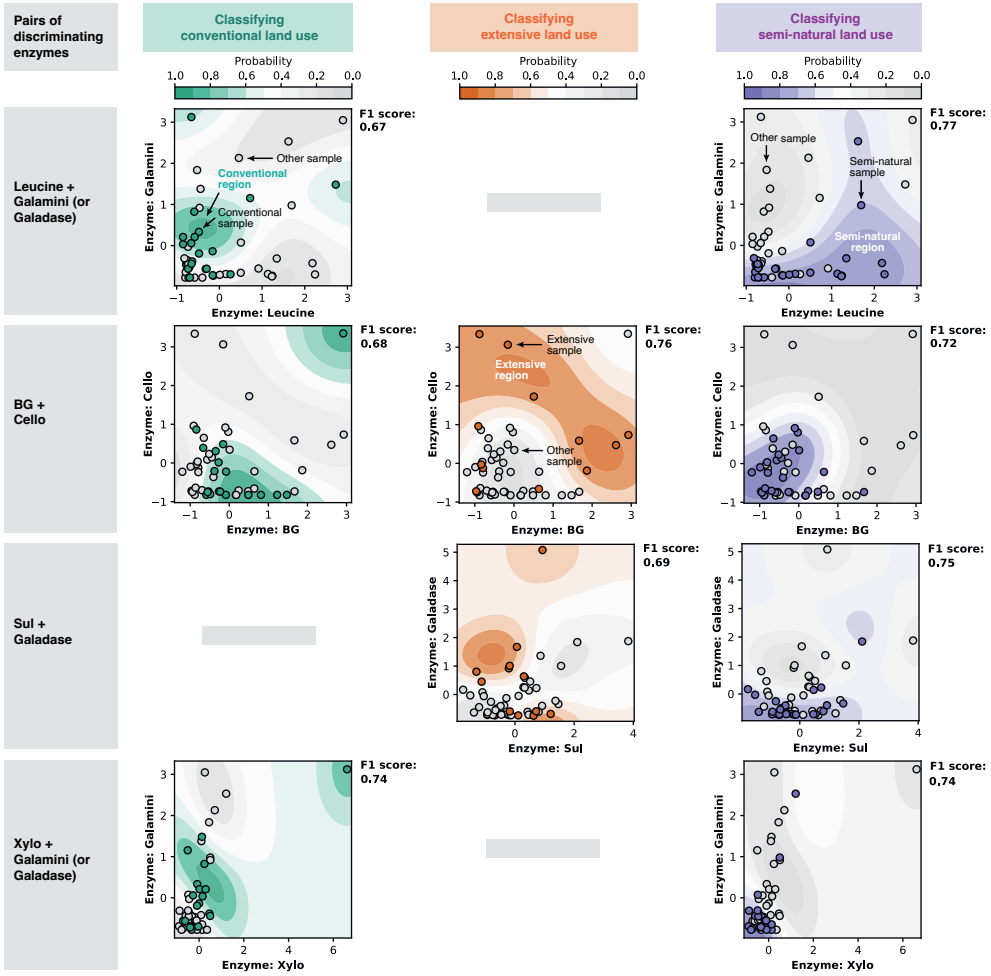


Figure S6.2: Classification of land-use guided by post-priori land-use labels, using enzymatic activity. Visual probabilistic classification maps for pairs of enzymes, across land-use types, when the pair can discriminate a land-use label from the others with F1 > 0.67. Each row represents the ability of the pair to distinguish one land-use type (column) from the other land-use types. The distributions are scaled (mean 0, variance 1). All field samples were used for classification and are overlaid, coloured by their post-priori categories. When the sample falls within the wrong type region, the sample classification disagrees with the post-priori label. Sample regions are non-linear.

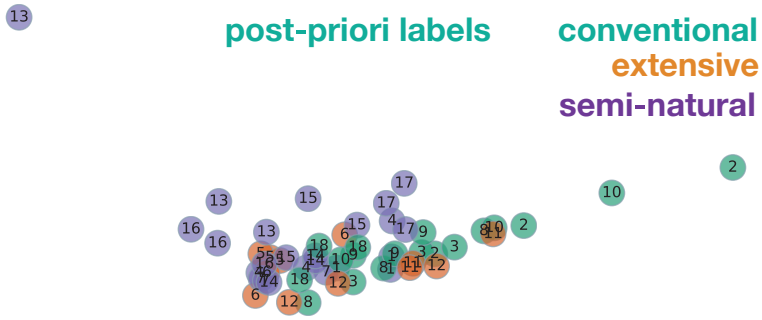


Figure S6.3: Principal component analysis (PCA) of volatile organic compound (VOC) profiles across samples. Points represent individual samples and are coloured according to post-priori land-use classification (conventional, extensive, semi-natural). Numbers indicate field identities, with three samples per field.

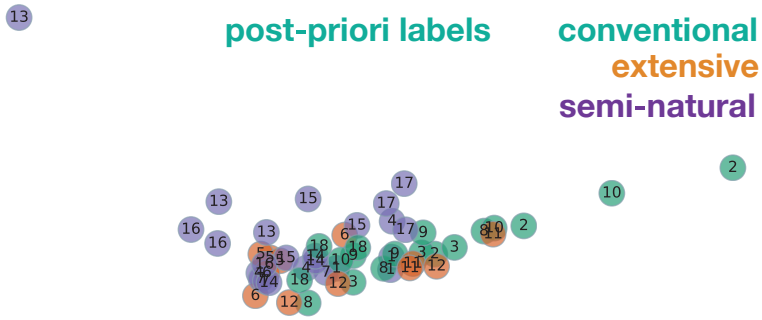


Figure S6.4: Principal component analysis (PCA) of soil enzyme activity profiles across samples. Points represent individual samples and are coloured according to post-priori land-use classification (conventional, extensive, semi-natural). Numbers denote field identities, with three samples per field.

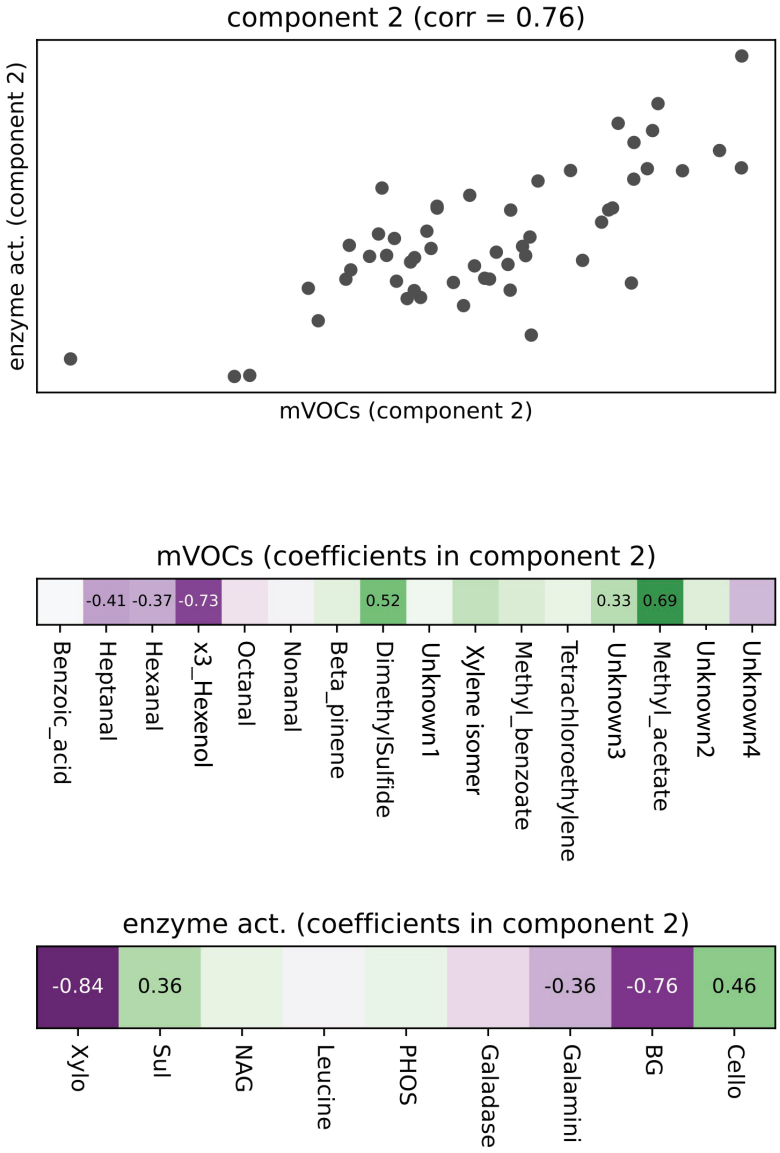


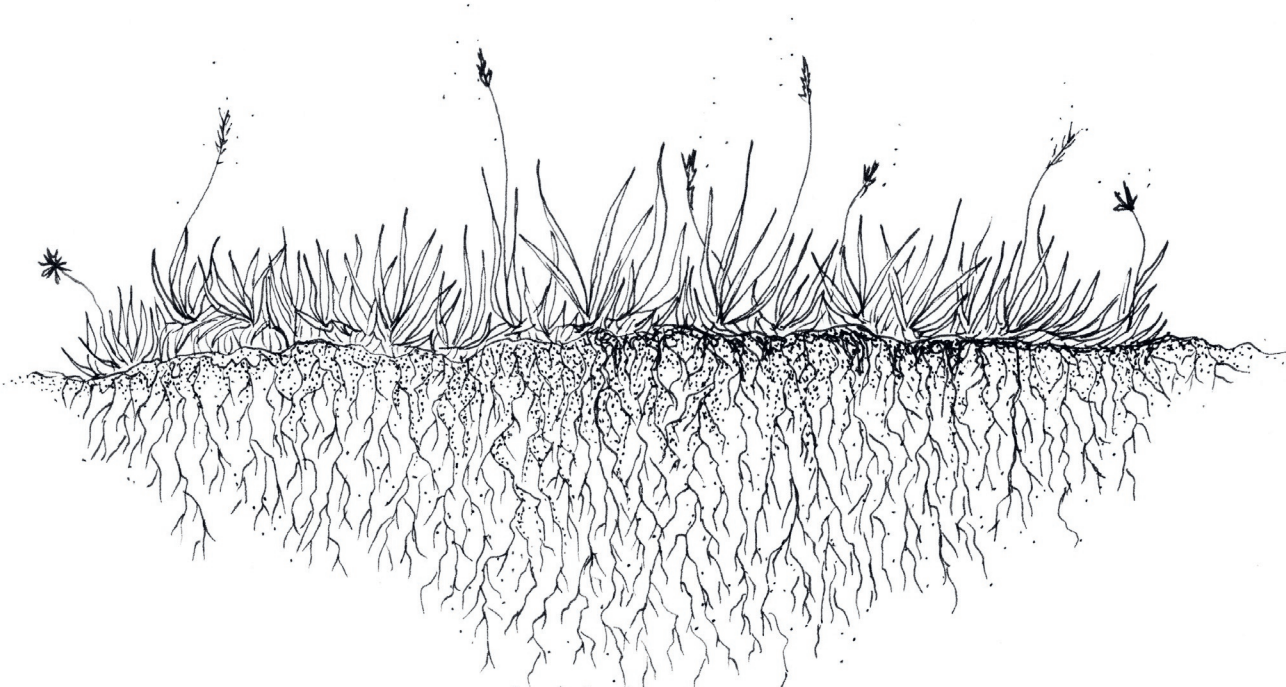
Figure S6.5: Second canonical axis of Canonical Correlation Analysis (CCA) between soil extracellular enzyme activities and microbial volatile organic compounds (mVOCs). The scatter plot displays paired canonical scores across all 54 samples (r = 0.76), with each axis representing a weighted composite of enzyme activities (left heatmap) and mVOCs (bottom heatmap), respectively. The second canonical axis captures opposing patterns within each dataset, representing variation not explained by the dominant shared gradient shown in Figure 6.4. Heatmap coefficients indicate the weights assigned to each variable; coefficients with absolute values < 0.33 are omitted for clarity but all variables were included in the analysis. Positive coefficients (green) indicate variables that increase along the canonical axis; negative coefficients (purple) indicate variables that decrease. Enzyme abbreviations: Xylo = xylosidase, Sul = sulfatase, NAG = N-acetyl-glucosaminidase, PHOS = phosphatase, BG = β-glucosidase, Cello = cellobiohydrolase.



Photograph taken at the author's family farm in Zeeland, the Netherlands.

7

Soil inoculation for enhancing agricultural grassland conversion



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In preparation

Abstract

Restoring species-rich grasslands on agricultural land requires recovering both plant and soil communities simultaneously, yet soil biotic communities recover slowly and can constrain the rate of vegetation recovery through plant-soil feedbacks. Whole-soil inoculation offers a means to actively accelerate this process by steering soil community composition toward that of a species-rich donor grassland. The transfer of soil from a donor grassland to a degraded recipient site, known as whole-soil inoculation, offers a means to actively steer soil community composition, but its effectiveness under agricultural conditions remains poorly understood. We combined a controlled greenhouse experiment and a two-year field experiment to investigate whether whole-soil inoculation induces detectable shifts in microbial community composition and functional group structure during early stages of grassland conversion. In the greenhouse, inoculum origin and seed mixture both significantly affected microbial community composition, with containers receiving donor soil developing communities most similar to the original donor, confirming that living biota drove community shifts. In the field, however, no detectable inoculation signal emerged in microbial community composition, regardless of seed mixture. Instead, sampling period explained the majority of variation in soil microbial community composition, reflecting gradual microbial changes during the conversion from arable to grassland. Both prokaryotic and fungal communities shifted directionally towards the donor community over time in all plots, independently of inoculation, suggesting that seed mixture also had a significant part in driving the belowground changes. Inoculation also did not lead to detectable shifts in microbial functional group composition in either the greenhouse or field experiment, although functional guilds of the microbial community changed in abundance over time in the field. We discuss methodological constraints, including thin inoculum layers, insufficient prior disturbance, and short duration of the soil monitoring that may have limited detection of inoculation effects. Our results suggest that under realistic agricultural conditions, whole-soil inoculation in combination with sowing is insufficient to redirect microbial community assembly two years after inoculation. Hence longer monitoring periods, larger inoculum quantities, and integration with topsoil management are needed to further improve the potential of inoculation-based grassland restoration.

7.1 Introduction

In Europe, agricultural intensification has driven the widespread loss of species-rich grasslands (Bardgett et al., 2021; Hempel et al., 2025), largely through conversion of herb-rich communities into perennial ryegrass dominated systems. These changes reduce aboveground biodiversity, but also alter belowground communities, as shifts in vegetation structure influence soil biota, modifying the belowground community composition and affecting key soil functions such as nutrient cycling, decomposition, and soil fertility (Bardgett & van der Putten, 2014; Creamer et al., 2022). Critically, soil community composition and plant performance are linked through plant-soil feedback (PSF): plants modify the soil environment, which reciprocally determines plant performance, positively or negatively (Bever, 1994; Bever et al., 1997). Intensification of grasslands can disrupt the coupled dynamics between plants and soil organisms that sustain ecosystem functioning (Chomel et al., 2022). Previous studies have shown restoring these grasslands is not merely a matter of re-establishing plant communities but also requires recovering of the belowground biodiversity that interacts with plant community diversity and abundance (Wubs et al., 2016; Wubs et al., 2019a).

Restoration towards species-rich grasslands is a notoriously slow process (Bakker & Berendse, 1999; Van Diggelen et al., 2001), constrained by high residual nutrient levels and by the plant-soil feedbacks, which can arrest degraded systems into an early-secondary successional state (Kardol et al., 2006; Van Der Putten et al., 2013). While plant communities can be steered relatively quickly through seeding, soil communities recover more slowly as legacy effects in the soil can have long-lasting effects that may prevent the belowground community development (Elgersma et al., 2011; Heinen et al., 2020). To accelerate this process, the transfer of soil biota from a reference grassland by means of whole soil inoculation offers a means to actively steer the soil community by overcoming dispersal limitations (Gerrits et al., 2023; Robinson et al., 2025). Soil inoculation has been proven to be successful in steering above and belowground communities towards the reference ecosystem (Han et al., 2022; Wubs et al., 2016). Whole soil inoculation involves transferring topsoil from a donor site to a receiving site (Gerrits et al., 2023). The translocated soil contains resident plant material, such as seeds, roots, and rhizomes, as well as a whole range of soil biota (Harris & Van Diggelen, 2008). By introducing new species, the inoculant can change the present soil community. Once established, these organisms can steer plant community development through plant-soil feedbacks, with outcomes depending on the balance between symbiotic mutualists (e.g. arbuscular mycorrhizal fungi) and antagonists, such as pathogens and root-feeding nematodes (Heinen et al., 2020; Kardol et al., 2006). Success of restoration by inoculation is typically defined as the

convergence of the recipient plant community toward that of the donor site (Wubs et al., 2016). While inoculation has shown promise in natural and semi-natural ecosystems, whether it performs equally well in agricultural grasslands, where a legacy of intensive management may impede the establishment and persistence of introduced soil biota, remains overlooked. There are two potential barriers that may limit the success of inoculation in agricultural grasslands due to past land management. First, biotic resistance from the present soil community might prevent introduced species from establishing, as competition from the already established soil community can suppress newly introduced species (Mallon et al., 2015; O'Callaghan et al., 2022; Raaijmakers & Mazzola, 2016). The effectiveness of the inoculated microbial consortia as favouring mid- and late-successional plant species, while suppressing early successional dominants (Wubs et al., 2019b) may depend on the presence of a resident soil community (de Deyn et al., 2004; Wubs & Bezemer, 2015). Second, legacy soil conditions from prior management, most notably elevated nutrient availability, have been shown to impede the establishment of inoculated soil biota (Gerrits et al., 2023; Robinson et al., 2025). This has been demonstrated in several studies that point at legacy effects in soil conditions from previous management, which act as barriers for microbial establishment. Specific groups of soil microbes, such as rhizobia and arbuscular mycorrhizal fungi, are known to be negatively influenced by high levels of nitrogen (Ceulemans et al., 2019; Nishida, 2018). Therefore, high nitrogen levels due to past fertilisation can hamper the establishment and persistence of soil biota. Beyond establishment success and steering of the soil community, it remains uncertain whether soil inoculation can also restore functionality of the soil community (Kardol & Wardle, 2010). This is important as soil microbes are known to be key drivers of almost all soil functions (Bardgett & Van Der Putten, 2014; Creamer et al., 2022). In agricultural soils, intensification has shown to suppress symbiotic mutualists communities while favouring pathogens (Lekberg et al., 2021). Inoculation with extensively managed donor soil may reintroduce functional guilds that enhance nutrient turnover and soil fertility (Román et al., 2018). Inoculation studies have shown evidence that inoculation, besides composition, can alter microbial abundance and activity and may introduce key taxa that have not naturally colonised degraded sites (Han et al., 2022). Soil inoculation may therefore help overcome dispersal barriers for microbial groups that regulate key functions such as decomposition, nutrient mineralisation and nitrogen cycling.

7.1.1 Evidence from controlled and field studies

Experimental evidence shows that the outcomes of inoculation may vary across environments. Under controlled conditions, applying an inoculant to sterilised soils generally

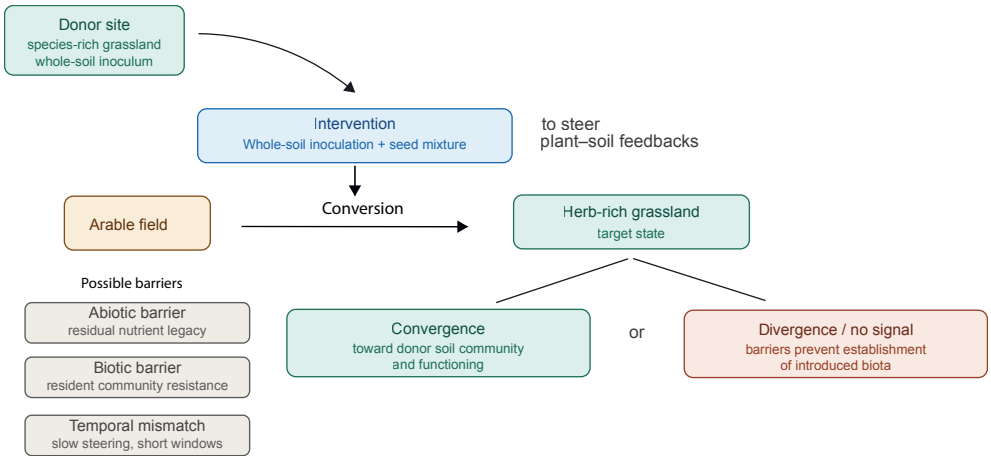


Figure 7.1: Conceptual framework illustrating the study rationale and experimental design. A donor site (species-rich grassland) provides whole-soil inoculum and seed mixture, which are applied as an intervention to convert an arable field toward a herb-rich grassland target state. Following conversion, two outcomes are possible: (1) convergence, where the soil community and functioning shift toward that of the donor grassland, or (2) divergence or no signal, where barriers prevent the establishment of introduced biota. Three categories of potential barriers to successful conversion are identified: abiotic barriers (residual nutrient legacy), biotic barriers (resident community resistance), and temporal mismatch (slow steering and short observation windows).

shows strong and rapid responses in soil fauna and plant community composition (De Deyn et al., 2003, 2004; Gerrits et al., 2023). Field studies report slower and more variable responses. Studies have reported that the trajectory of inoculation can be dependent on the removal of the topsoil where most microbial activity takes place is important, or the inoculum quantity, origin or type (Gerrits et al., 2023; Han et al., 2022; O'Callaghan et al., 2022; Wubs et al., 2016). As an example of the influence of type; whole-soil inoculation introduces a heterogeneous community that may support more durable functional recovery than synthetic inocula composed of a few strains (Emam, 2016a; French et al., 2017). As a result of the variability in response, previous studies have underlined two knowledge gaps that hamper predicting the success of soil inoculation in the field. First, there is often a time lag before shifts in soil communities become detectable, ranging from several years in disturbed soils to nearly a decade in intact agricultural soils (Wubs et al., 2016). Second, the longevity of inoculum effects remains uncertain: introduced communities may fade, stabilise, or transform depending on local soil conditions and experimental set-up. Understanding early stages of microbial establishment is therefore crucial for predicting long-term restoration trajectories.

7.1.2 Study rationale and aims

Our aim was to investigate whether soil inoculation can induce detectable changes in soil microbial community composition during the early stages of grassland conversion in an agricultural setting (Fig. 7.1). We combined a controlled greenhouse experiment and a field experiment using soils and seed mixtures from the same sites. In the greenhouse, we experimentally tested inoculation effects under standardised conditions, comparing living and sterilised soils from degraded and donor grasslands to determine the biotic potential of inoculation. In the field experiment, we evaluated whether belowground shifts occur, similar to the greenhouse experiment, under realistic agricultural management using different herb-rich seed mixtures.

We focused on the establishment and persistence of inoculated microbial communities, and on shifts in microbial functional groups, as these represent the foundation for long-term plant-soil feedback and functional restoration. Specifically, we asked:

1. Does inoculation lead to detectable shifts in microbial communities in controlled lab situations and in the field, and are these shifts consistent across seed mixtures?
2. Which microbial taxa or guilds are most indicative of successful inoculation in the lab and in the field?
3. Does inoculation lead to detectable shifts in microbial functional groups in controlled situations and in the field?

We tested the hypothesis that inoculation leads to detectable shifts in microbial community composition under both controlled and field conditions, but that the effects are strongest under controlled conditions. We also tested the hypothesis that more herb-rich seed mixtures will enhance the inoculation effect due to more intensive plant-soil feedbacks. We also tested the hypothesis that taxa associated with extensive herb-rich grasslands, for example arbuscular mycorrhizal fungi and saprotrophic decomposers, will serve as indicators of successful inoculation, showing greater establishment and relative abundance in inoculated soils than the control. Finally, Inoculation will increase the abundance of functional groups involved in decomposition, nutrient mineralisation, and nitrogen cycling, consistent with functional traits associated with species-rich donor soils.

7.2 Materials and methods

7.2.1 Greenhouse experiment

Experimental design

To isolate the effect of the inoculant on the present microbial community composition, we first established a controlled greenhouse experiment. We compared microbial community assembly in containers after inoculation with living and sterilized (control) inocula. The experimental design was fully factorial with two factors: inoculum type (four levels) and seed mixture (three levels). Containers were filled with sterilised field soil (4.9 kg of moist weight) after which an inoculum (0.1 kg) was added (98% soil; 2% inoculum). Each 5 Liter pot of 17 x 17 x 17 cm contained one of the four following inoculum treatments:

1. Field soil
2. Sterilised Field soil
3. Donor soil
4. Sterilised Donor soil

Each inoculum treatment was crossed with three seed mixtures representing common types of agricultural grasslands in the region: 1) monoculture; MC (100% Lolium perenne BG3 Diploid), 2) extensive herb-rich; EHR (Biodivers "Kuikenland B145" mixture) and 3) productive herb-rich; PHR (Pure Graze "Saladebuffet doorzaai" + Lollium perenne BG3 Diploid). As a control, we also included a treatment where no plants were sown in order to test whether sowing itself affected the microbial communities. Each combination was replicated six times (n = 6) and randomly arranged within blocks.

Soil collection and preparation

Field soil was collected from the site where the inoculation experiment was carried out. Donor soil was collected from a species-rich Arrhenatheretum elatiori grassland in the Amerongse Bovenpolder (51°59'26.5"N 5°28'15.7"E), The Netherlands that was managed by Staatsbosbeheer (Dutch State Forestry Service). The donor site had a silty (clay) loam soil texture that differed from the sandy loam texture of the experimental field site (bodem-data.nl), but it is the original source of the seed mixture used for the extensive herb-rich treatment (Biodivers "Kuikenland B145").

Donor soil was collected within a randomly selected 2 x 2 m^2 square, from 5-10 cm below the soil surface, after removing the 5 cm of topsoil to remove the majority of seeds,

rhizomes, and litter. At the field site, soil was collected from two plots of 9 m² at the edge of the experimental area. From these collected samples, soil was used for sterilisation with γ-irradiation at minimally 25 kGy (STERIS, Ede, NL). Sterilisation success was confirmed by incubating sterilized soil samples for 24 hours at agar plates in an incubator (dark room, 25 °C); six bags with sterilized soil showing contamination were excluded. All soils were stored at 4 °C until use. Because long storage times can alter soil biotic communities, field soil and donor soil were collected from the exact same locations (using a dGPS, Topcon), one day before the start of the greenhouse experiment to serve as "living" inocula.

At the start of the experiment, containers were cleaned with ethanol, and the filling of the containers was done with ethanol-cleaned tools to avoid cross-contamination. Containers (96 in total) were filled with sterilised field soil and inoculated with either field soil (F), sterilised field soil (S-F), donor soil (D), and sterilised donor soil (S-D) at a 98:2 ratio (w/w). Containers were sown with one of the seed mixtures: MC (0.1215 g), EHR (0.0810 g), or PHR (0.1620 g; Table 7.1), or left unsown. After sowing, containers were watered with rainwater, covered with plastic foil for three days to promote germination and then watered weekly by filling the saucers beneath each pot. This allowed water to be absorbed from below until no further uptake occurred ensuring uniform soil moisture while avoiding surface disturbance. Because water uptake depended on plant biomass, containers with less vegetation absorbed less water than those with denser plant cover. Weeding was carried out as needed to remove moss and excessively dominant individuals. When a species threatened to overshadow or suppress neighbouring plants, individual plants were removed to prevent competitive exclusion. This was particularly relevant for Cichorium intybus (in the PHR seed mixture), which can develop disproportionately large biomass in small containers. Aboveground biomass (shoots) was harvested eight times over nine months. After nine months, the top 10 cm of soil from each container was collected and sieved (2 mm). Subsamples of 1 g were stored at -20 °C, whereas 100 g was stored at 4 °C (field -moist conditions), and another 100 g was dried at 40 °C and 105 °C for moisture determination. The 105 °C-dried soil was subsequently ashed at 550 °C for loss-on-ignition analysis. Further soil processing followed the same procedures used for the field experiment samples (see below).

7.2.2 Field experiment

Experimental Design

Parallel to the greenhouse study, we carried out a field experiment to assess how soil inoculation with donor grassland soil would alter microbial communities under real

Table 7.1: Species composition of different seed mixtures used for experimental set-up.

Seed mixture	Abbreviation	Species
Extensive herb-rich mixture (B145 Kuikenland, Biodivers)	EHR	*Alopecurus pratensis*, *Achillea millefolium*, *Silene flos-cuculi*, *Cerastium fontanum*, *Poa trivialis*, *Trifolium dubium*, *Lotus pedunculatus*, *Cardamine pratensis*, *Anthoxanthum odoratum*, *Festuca rubra*, *Trifolium pratense*, *Ranunculus acris*, *Plantago lanceolata*, *Hordeum secalinum*, *Lathyrus pratensis*, *Rumex acetosa*, *and Vicia cracca*
Productive herb-rich mixture (2/3 Pure Graze "Saladebuffet doorzaai", 1/3 *Lolium perenne*)	PHR	*Sanguisorba minor*, *Cichorium intybus*, *Petroselinum crispum*, *Plantago lanceolata*, *Achillea millefolium*, *Carum carvi*, *Onobrychis viciifolia*, *Trifolium repens*, *Trifolium pratense*, *Medicago sativa*, *Anthyllis vulneraria*, *and Lolium perenne* (BG3 Diploid)
Control mixture (monoculture)	MC	*Lolium perenne* (BG3 Diploid)

agricultural practice (Fig. 7.2). The experimental field was located in Groesbeek, the Netherlands (51°46'42.0"N 5°56'05.2"E), and consisted of loess soil classified as sandy loam (bodemdata.nl). The field was a former conventional grassland (2015-2019) that had been converted to arable use for the previous three years, during which maize (2020), onions (2021), and potatoes (2022) were grown. This grassland is part of a practical rotational system, where grassland phases alternate with arable crops every four to five years. The conversion to an arable phase creates a disturbance through ploughing, which can reduce organic matter and nitrogen and a restructuring of the microbial community. By disrupting the existing community, this disturbance may create a window of opportunity for the establishment of new microbial communities.

The field was divided into six experimental blocks of 9 × 6 m^2 each, each separated by a 0.5 m buffer zone (Fig. S7.1). Each block was further divided into six 3 x 3 m^2 plots, with 20 cm-wide border lanes in between to separate treatments. Within each plot, only the inner 2 x 2 m^2 was used for sampling of vegetation and soil. For each block, we have sown three seed mixtures with or without inoculation (six treatments in total). The three seed mixtures corresponded with the mixtures used for the greenhouse experiment (see Table 7.1). Prior to the experiment, the field was mechanically ploughed and harrowed with a rake. In late September 2022, baseline soil samples were collected before applying any experimental treatments. Within each block, twelve soil cores (0-25 cm depth) were taken and pooled into one composite baseline sample per block ("baseline").

Soil Inoculation

In October 2022, soil inoculum was collected from eight random sites at the Amerongse Bovenpolder. At each site, 1 m^2 of soil was excavated. The top 0-5 cm sod layer was re-

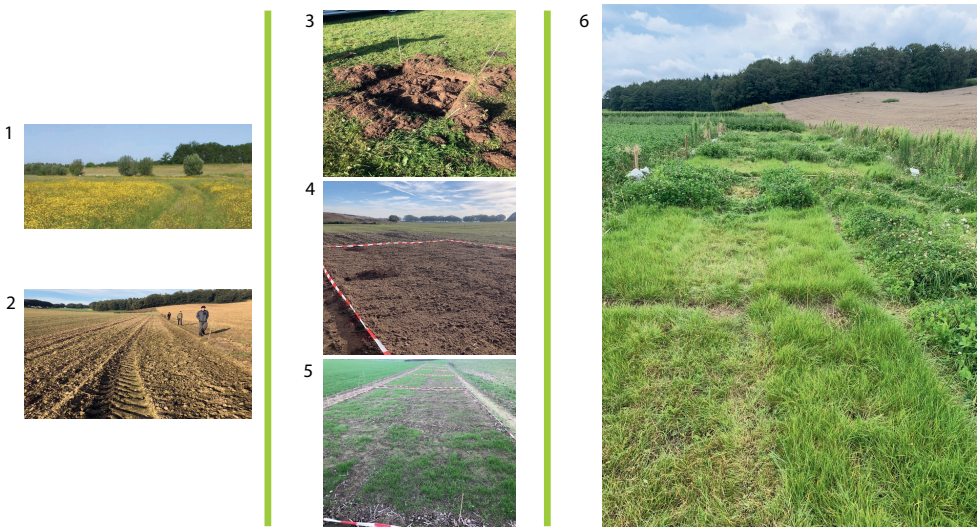


Figure 7.2: Photos of experimental set-up in the field. 1) Picture of the species-rich grassland from the donor site in the Amerongse Bovenpolder (Staatsbosbeheer, n.d.), 2) Recipient site (rotational grassland temporarily converted to arable land)), before the inoculation and other preparations, 3) example of a sampling spot for collecting the donor soil, 4) Recipient site after preparations, with inoculation soil pile in the left corner, 5) experimental field-site after 8 weeks of inoculation, 6) inoculation site at the first harvest.

moved, after which the 5-10 cm layer was collected, resulting in 80 L of inoculum soil. The inoculum was manually sieved to remove stones and coarse roots to minimise disturbance, stored outdoors overnight under rain-protecting cover (with a night temperature of 3.3 °C). The following day, in each block, the inoculum soil was applied to half of the randomly selected plots at a rate of 2.5 L $m^{-}2$, corresponding arithmetically to a 2.5 mm layer. The three seed mixtures were then sown at rates of; 1) EHR (Kuijkenland, BIODIVERS: 30 kg seeds/ha), inoculum, 2) PHR (40 kg/ha): composed of 2/3 PureGraze "Saladebuffet doorzaai" and 1/3 Lolium perenne (BG3 Diploid) and 3) MC: 100% Lolium perenne (BG3 Diploid) at 20 kg/ha (Table 7.1). To check for even seed distribution, seeds were mixed with 1.5 L of 'silver sand' per plot. After sowing by hand, the soil surface of all plots was pressed by a manual roller to enhance germination by promoting seed-soil contact.

Field Management

In March 2023 and 2024, to mimic agricultural practice, plots were fertilized with dried cattle manure pellets (50 kg N/ha or 5 g N per m^2) and kieserite (100 kg/ha; 50% SO_3 and 25% $MgSO_4$). Due to drought conditions in 2023, irrigation was applied once using a hose reel system. Water inputs were monitored using rain gauges, and post-event water volumes were assessed to ensure variation between plots did not exceed two standard deviations

(Table S7.1). To prevent cross-contamination between plots, the lanes between plots were regularly weeded. Residual potato plants from previous cropping were removed from the experimental field (plots and lane). Communication with neighbouring farmers ensured that their management practices (e.g., pesticide use, irrigation) did not affect the field experiment.

Biomass Collection

In 2023 and 2024, during the growing season, from late April to late October, plots were mown five and four times, respectively. Only the central 2 × 2 m^2 area was used for biomass collection to prevent edge effects. Each plot was mown in a single pass (back and forth) using a 90 cm fixed-bar automated mower. Fresh biomass was collected immediately in plastic bags, and stored at 4°C. After biomass collection, the remaining vegetation from the buffer zones was mown and removed from the plots. Within three days, biomass samples were homogenized, and a 250 g subsample was sorted into grasses, legumes, and other herbs. Each category was dried at 70°C until constant weight. Total dry biomass per plot was calculated by summing the three fractions and expressing the result per unit area (mowed area) (kg ha^{-1}).

Vegetation Surveys

In October 2023 and August 2024, plant community composition was assessed using the Braun-Blanquet method in the inner 2 x 2 m^2 area of each plot. Species richness per plot was calculated as the total number of species, while plant diversity was assessed using the Shannon diversity index.

Soil Sampling and Processing

To avoid edge effects, all soil samples were collected from the central 2 × 2 m^2 of each plot. To detect short-term effects of the inoculation, the first soil sampling occurred eight weeks after inoculation (November 2022) in the top layer of the soil. From the upper 0-5 cm soil layer, five subsamples were collected per plot using a 2 cm diameter soil corer. These samples were pooled into one composite plot sample. For every treatment, there were 6 replicate composite samples. Samples were stored at 4°C during transport, sieved using 2 mm mesh size, and homogenized, all in the same day. A subsample of 1 g was stored at −20°C in Eppendorf tubes for DNA analysis. In October 2023 and 2024, from each 2 × 2 m^2 plot, subsamples were collected by taking 12 cores from 0-25 cm depth and pooled to one composite sample. Pooled soil sampled were then transported (4°C). In the lab, the pooled sam-

ples were sieved within one week (at a mesh size of 2 mm), and split into subsamples for different analyses: 200 g of field moist soil (stored at 4°C) and 100 g of air-dried soil (dried at 40°C for 48 h) for additional chemical analyses and 1g of frozen soil (stored at –20°C) for DNA analyses. From each field-moist subsample, 20 g was used to determine moisture content (dried at 105°C for 24 hrs) and organic matter content (loss on ignition at 550°C for four hrs). Nutrient analysis followed Boone et al. In summary, NaCl extractions from field-moist subsamples were used to determine soil acidity, NO_3^-, NH_4^+ and PO_4^{3-} (Auto Analyzer 3, Bran+Luebbe) and elemental concentrations of Al, Ca, Fe, K, Mg, Mn, P, S (ICP-OES, iCAP 6000, Thermo Fisher Scientific). Plant-available phosphorus was quantified using the P-Olsen method (Olsen et al., 1954) using the air-dried subsamples. Total Carbon (C) and Nitrogen (N) content were analysed using an elemental analyser (NA1500, Carlo Erba, Thermo Scientific) on 20-30 mg of air-dried, ground material. Soil organic carbon (SOC) was determined by treating the air-dried grounded samples with HCl, to acidify the inorganic carbon content present in the sample. After treatment, samples were measured with an elemental analyser (NA1500, Carlo Erba, Thermo Scientific) on 30-40 mg of the acidified material.

7.2.3 DNA analysis

Both the greenhouse samples and field experiment samples were extracted, processed and sequenced in one batch. Soil microbial DNA was extracted as described by Boone et al. (2025). In short, from each sieved soil sample, 0.25 g was processed using the DNeasy PowerSoil Pro Kit (Qiagen, China) and subsequently purified with a gDNA Clean-Up Kit (Qiagen). Final extracts were eluted in 50 µL of elution buffer and transported on dry ice to Novogene (Cambridge, UK) for amplicon library preparation and sequencing. Prokaryotic communities (bacteria and archaea) were targeted by amplifying the V4 region of the 16S rRNA gene using primers 515F/806R (Caporaso et al., 2012), while fungal communities were profiled by amplifying the ITS2 region with primers ITS3-2024F/ITS4-2409R (White et al., 1990). Each PCR reaction consisted of 15 µL Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 2 µM forward primer, and approximately 10 ng of template DNA. The cycling conditions were: initial denaturation at 98 °C for 1 min; 30 cycles of 98 °C for 10 s, 50 °C for 30 s, and 72 °C for 30 s; followed by a final extension at 72 °C for 5 min. PCR products were visualised on 2% agarose gels, pooled in equal molar amounts, and purified using the Universal DNA Purification Kit (TianGen, China). Sequencing was performed on an Illumina NovaSeq 6000 platform (250 bp paired-end reads; Novogene, Cambridge, UK). Adapter sequences, barcodes, and primers were removed by the sequencing provider before bioinformatic processing. Quality filtering and sequence inference were carried out

in DADA2 (v1.8; Callahan et al., 2016), with no ambiguous bases permitted. For 16S amplicons, both forward and reverse reads were truncated at 218 bp; ITS2 reads were filtered with a minimum length threshold of 50 bp. To address the binned quality scores characteristic of NovaSeq data, we applied a modified error estimation function using local regression ('loess') with a maximum error rate of 0.25. Chimeric sequences were removed using the consensus method. Taxonomic assignments were made against the SILVA SSU database (v138, 2019) for 16S sequences (Quast et al., 2012) and the UNITE database (v9.0) for ITS2 sequences (Kõljalg et al., 2013; Nilsson et al., 2019). Amplicon sequence variant (ASV) tables produced in DADA2 were analysed in the Microeco package (Liu et al., 2021). All ASVs that could not be taxonomically assigned were excluded to avoid potential inclusion of non-microbial sequences. To standardise sequencing depth across samples, reads were rarefied to 10,000 per sample. Before rarefaction, sequencing depth ranged from 11 to 27,962 for the 16S dataset and from 2,378 to 44,584 reads per sample for the ITS2 dataset. This step removed 13995 taxa from the 16S dataset and 600 taxa from the ITS2 dataset.

7.2.4 Data analysis

Vegetation composition

Plant community composition data from 2023 and 2024 were analysed separately using non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities. Species abundance data were square-root transformed prior to analysis to reduce the influence of dominant taxa. Bray-Curtis distance matrices were computed using the vegan package in R (Oksanen et al., 2025). Homogeneity of group dispersions was tested using the betadisper function followed by permutation tests (permutest) to assess whether multivariate dispersion differed among treatment groups. For the 2023 dataset, dispersion differed significantly among treatments. Ordinations were performed using the metaMDS function with two dimensions (k = 2) and up to 100 random starts (trymax = 100) to ensure stable solutions. Treatment ellipses representing 95% confidence intervals were added to NMDS plots to visualise group separation. To test for differences in plant community composition among treatments, permutational multivariate analysis of variance (PERMANOVA) was conducted using the adonis2 function, with treatment as a fixed factor and block as a random blocking factor. The permutation design was specified using the how() function in vegan to restrict permutations within blocks. Because each treatment had only two replicates per block, within-block restricted pairwise permutations were not feasible. Therefore, pairwise PERMANOVA tests were performed without block restriction using the pairwiseAdonis package (Martinez, 2020) and were interpreted descriptively.

Microbial community analyses

Microbial community analyses for both the greenhouse and field experiments were conducted using the microeco R package (Liu et al., 2021). To reduce the influence of highly abundant taxa, ASV counts were log-transformed [log(x + 1)] prior to analysis, and a new microtable object was constructed from the transformed ASV tables. Bray-Curtis dissimilarities were calculated from the log-transformed data (trans_beta$new, measure = "bray"). Ordinations were performed using principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarities (cal_ordination, method = "PCoA"). The first and second axes explained the largest proportions of variation, but combinations of the first and third axes often revealed clearer treatment-related separation, highlighting compositional shifts associated with inoculation. To test whether live and sterilised inocula from the field and donor affect prokaryotic (bacteria and archaea) and fungal community composition under controlled conditions, we analysed the microbial community in the soil from the containers of the greenhouse experiment. To assess whether sowing influenced microbial community composition, we first compared sown and unsown containers using PERMANOVA (adonis2, 999 permutations; Anderson, 2017). Because sowing had a significant but relatively small effect (ITS2: R^2 = 0.06, p = 0.001; 16S: R^2 = 0.01, p = 0.001), unsown containers were excluded from subsequent analyses to ensure comparability with the field experiment, in which all plots were sown.

Community dissimilarities among inoculation treatments were then tested using PERMANOVA with inoculation treatment as the fixed factor and Replicate as the blocking variable. Pairwise PERMANOVAs were performed for post-hoc comparisons. To identify ASVs introduced through inoculation, relative-abundance tables were converted to presence-absence matrices (presence = 1, absence = 0). Samples were merged by inoculation group (sterilised field, field, sterilised donor, donor and original donor), and ASVs were classified as "introduced" if they were present in donor and original donor groups but absent from Null, field, sterilised field and sterilised donor groups. To cross reference the "introduced" ASVs, Indicator species analyses were performed with the indicspecies package (Cáceres & Legendre, 2009), computing indicator values (IndVal.g) with 999 permutations. Genera significantly associated with donor inoculated treatments ($p < 0.05$) were cross-referenced with the original donor ASVs to identify taxa introduced by the donor inoculum.

The field dataset was analysed following the same data processing and transformation pipeline as the greenhouse experiment. Community composition was visualised using PCoA based on Bray-Curtis dissimilarities. The first and third PCoA axes provided clearer separation among inoculation treatments than the first two axes, revealing treatment-driven shifts in community structure. Differences in community composition were

tested using PERMANOVA (adonis2, 999 permutations) with Treatment, Seed mixture, and Year as fixed factors and Field block as a stratification factor to account for spatial structure. Both overall and pairwise PERMANOVAs were conducted. To further evaluate treatment effects while accounting for random variation among blocks and years, linear mixed-effects models were fitted to the first PCoA axis scores using the lme4 and lmerTest packages (Bates et al., 2015). Treatment was included as a fixed effect, and Block and Year were included as random effects. Presence-absence analyses were used to identify donor-derived taxa by merging samples by inoculation group (Baseline, Donor, Inoculated2023, Inoculated2024, Control2023, Control2024). ASVs were considered donor-derived if they occurred in Donor and Inoculated samples but were absent from Baseline and Control treatments. In addition, to test whether "introduced" ASVs were establishing in the Inoculated2023 and Inoculated2024, Differential abundance analyses were performed at the genus level using the taxaturn class of microeco (taxaturn$new, taxa_level = "Genus", filter_thres = 0). For each plot, genera that changed significantly the first year after inoculation and subsequent year (2023 and 2024) were identified using the Wilcoxon rank-sum test with p-value adjustment. Similar to the greenhouse study, indicator taxa were identified using indicspecies as described above, to cross reference the "introduced" ASVs.

For both the greenhouse and field experiment, we analysed the presence of functional groups of the microbial communities. Functional profiles were inferred for prokaryotic and fungal communities using FAPROTAX (Louca et al., 2016) and FUNGuild (Nguyen et al., 2016), respectively, within the microeco package. Functional group abundances were calculated as abundance-weighted percentages (cal_spe_func_perc, abundance_weighted = TRUE). Differences in functional group composition among treatments were tested using one-way ANOVA (trans_diff, method = "anova", group = "Treatment_Inoc").

7.3 Results

7.3.1 Greenhouse Experiment

Under controlled greenhouse conditions, both inoculum origin and seed mixture significantly affected microbial community composition (Fig. 7.3A & 7.3B, Table 7.2). For the prokaryotic community, inoculum treatment explained 5% of the variation (PERMANOVA; $p = 0.001$), with seed mixture accounting for an additional 4.8% ($p = 0.001$). For the fungal community, inoculum treatment explained 24.5% of the variation (PERMANOVA; $p = 0.001$), with seed mixture accounting for an additional 4.3% ($p = 0.001$). Both sterilised donor and sterilised field soil differed significantly from their non-sterilised counterparts (Table

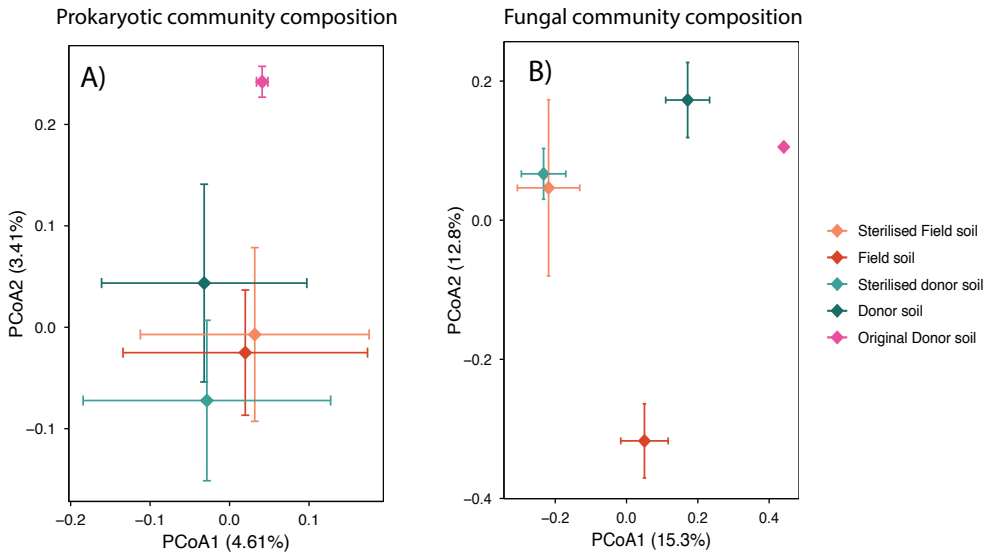


Figure 7.3: Principal Coordinate Analysis (PCoA) of A) 16s community, based on ASV's and B) ITS2 community from the greenhouse experiment, based on ASV's, grouped per treatment type. Sampling was done after nine months of development

7.2), indicating that living biota were the primary driver of community differentiation. Containers receiving fresh donor soil (D) developed prokaryotic and fungal communities most similar to the original donor, whereas sterilised donor soil (S-D) produced distinct communities (Fig. 7.3A & 7.3B).

To further characterise the composition of inoculation-associated communities, we identified ASVs uniquely introduced by the donor soil and absent in sterilised or control treatments: 78 prokaryotic and 94 fungal ASVs. Indicator species analysis revealed three prokaryotic genera (Blastococcus, Flavihumibacter, and an unknown genus) and ten fungal genera (Clonostachys, Staphylotrichum, Clohesyomyces, Slopeiomyces, Neopyrenochaeta, Ophiosphaerella, Hirsutella, Gaeumannomycella and Pyrenochaeta) significantly associated with the inoculation treatment and present in the donor soil, suggesting these taxa are candidates driving the observed community differences between treatments.

Table 7.2: Greenhouse experiment: Results from PERMANOVA analysis on Bray-Curtis dissimilarity matrix from 16S and ITS2 ASV's community composition for non-inoculated vs. inoculated treatments per year for the field (Inoculated8weeks vs Control8weeks, Inoculated2023 vs Control2023 and Inoculated2024 vs Control2024) and greenhouse experiment (Sterilised Field soil (S-F) vs Field soil (F) and Sterilised donor soil (S-D) vs Donor soil (D)). Significant differences (p < 0.05) are shown in bold.

Groups	S-F vs F	S-D vs D	Inoculated8weeks vs Control8weeks	Inoculated2023 vs Control2023	Inoculated2024 vs Control2024
Community	Adjusted p-value				
16S	**0.001**	**0.001**	1	0.465	0.66
ITS2	**0.001**	**0.001**	0.761	0.936	0.764

7.3.2 Field experiment

Plant community composition

One year after seeding, the plant communities from each seed mixture (Table 7.1) differed significantly from each other when accounting for the block effects (pairwise PERMANOVA; all pairs p = 0.001, Fig. 7.4A). These results demonstrate that plant community composition differed among treatments, although part of the variation may reflect differences in within-group variability. A test for homogeneity of multivariate dispersion (PERMDISP) indicated significant but moderate differences in within-group heterogeneity among treatments (F = 4.75, p = 0.015). Despite this, differences in community composition detected by PERMANOVA are unlikely to be driven solely by unequal dispersion, as group sizes were balanced and dispersion differences were small. Two years after seeding, composition of the plant communities appeared to converge, yet still being significantly different (pairwise PERMANOVA; all pairs p = 0.001, Fig. 7.4C). Regarding plant species diversity (Shannon index), the extensive herb-rich mixture (EHR) maintained the highest diversity in both years, although differences with the other treatments diminished over time (Figs. 7.4B and 7.4D). A notable exception was the MC+ plots in 2024, which showed both high forb biomass and high diversity. This pattern was driven by the invasion of buttercup (*Ranunculus acris*), a species present in the EHR seed mixture (Table 7.1) and therefore also likely present in the donor soil used in inoculated plots.

Soil community composition

Eight weeks after inoculation, for both prokaryotes and fungi, there was no significant difference in community composition between the inoculated and control plots (PERMANOVA; prokaryotes: F = 0.91, p = 1; fungi: F = 1.02, p = 0.383). However, both the control and inoculated plots for the prokaryotic and fungal communities had shifted slightly rela-

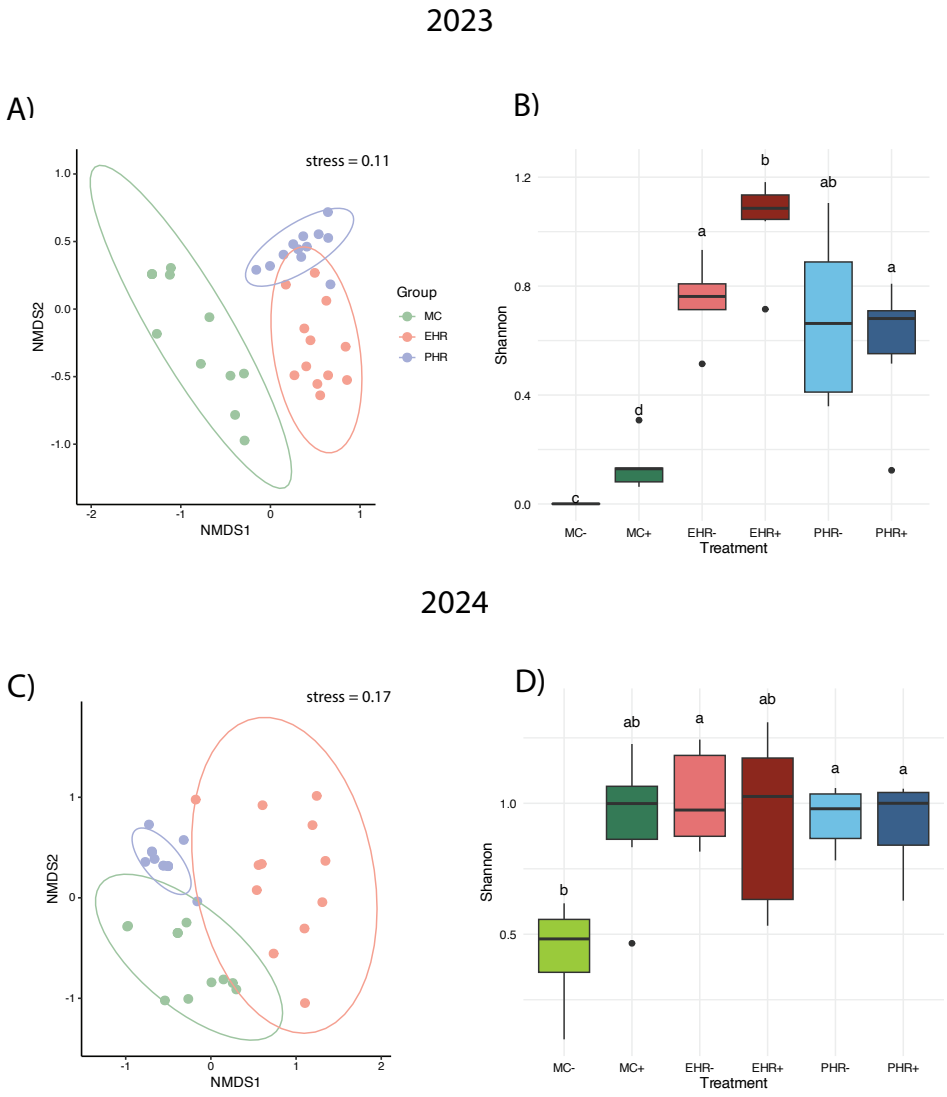


Figure 7.4: Field experiment: Composition and diversity of plant communities in the different treatments (Group); MC- = Monoculture not-inoculated, MC+ = Monoculture inoculated, EHR- = Extensive herb-rich mixture not-inoculated, EHR+ = Extensive herb-rich mixture inoculated, PHR- = Productive herb-rich mixture not-inoculated, PHR+ = Productive herb-rich mixture inoculated. A & C) Ordination based on share (percentage) of biomass per plant functional group (forbs, grasses and legumes for 2023 and 2024, B & D) Shannon diversity for plant species diversity for 2023 and 2024.

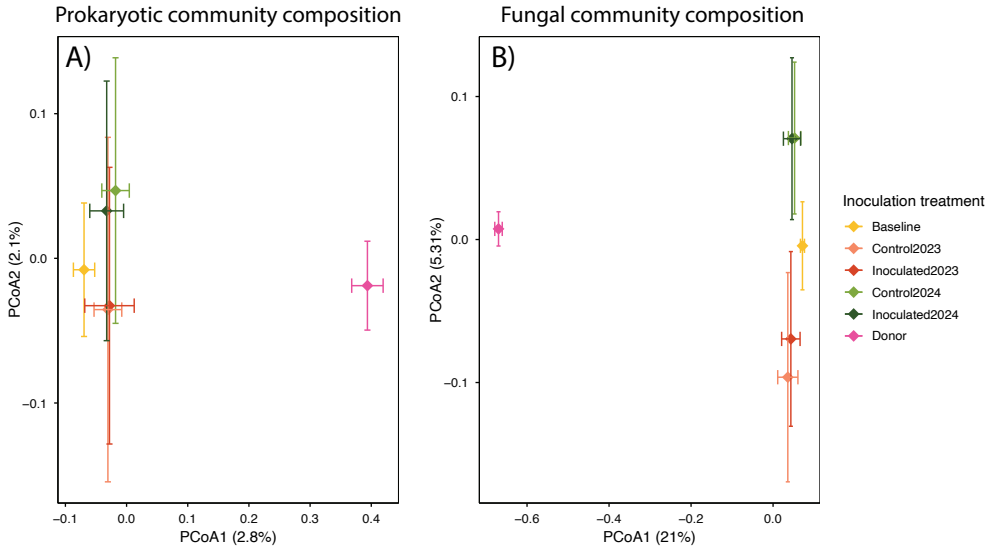


Figure 7.5: Field experiment: Coordinate Analysis (PCoA) of A) 16s community, based on ASVs and B) ITS2 community, based on ASVs, grouped per treatment type; Baseline = Receiving site before inoculation, Control2023 = non-inoculated plots after one year of inoculation, Inoculated2023 = inoculated plots after one year of inoculation, Control2024 = non-inoculated plots after two years of inoculation, and Inoculated2024 = inoculated plots after two years of inoculation.

tive to the baseline (Baseline) (Fig. S7.2A & S7.2B; Table S7.2 & S7.3), which was significant for the fungal community (PERMANOVA; F = 1.54, p = 0.006; F = 1.56, p =0.003), but not for the prokaryotic community (PERMANOVA; F = 1.04, p = 0.1; F = 2.06, p = 0.1).

One and two years after inoculation, the microbial communities in the inoculated plots did not differ significantly from those in the control plots (Fig. 7.5). Although all other group comparisons showed significant differences for both prokaryotes and fungi (adjusted p < 0.05), the control and inoculated plots within the same sampling year remained statistically indistinguishable (Tables S7.2-S7.3). The prokaryotic community was affected by a three-way interaction among inoculation treatment, seed mixture, and sampling period, however, this interaction explained only 2.4% (R^2 PERMANOVA, p = 0.318) of the variation in prokaryotic community composition. When testing the effects separately, neither inoculation treatment, nor seed mixture significantly influenced prokaryotic community composition (PERMANOVA, R^2 = 0.012, p = 0.505, R^2 = 0.024, p = 0.212, respectively). In contrast, sampling period showed a significant effect (p = 0.001) but only accounted for only 1.4% of the variation.

The fungal community showed a similar trend as the prokaryotic community composition (Fig. 7.5B). The three-way interaction (inoculation treatment, seed mixture, and sampling period) explained only 1.7% of the variation in fungal community composition

and was not significant. Again, when testing these effects separately, neither inoculation treatment nor seed mixture significantly influenced fungal community composition (PERMANOVA; R^2 = 0.0001, p = 0.780; R^2 = 0.022, p = 0.89, respectively), although sampling period accounted for 4.6% of the variation (p = 0.001). Based on presence-absence, we identified 143 ASVs for 16S and 43 ASVs uniquely introduced by the donor soil that were absent in Baseline and controls (non-inoculated plots). However, none of these showed significant increases in abundance across one year and two years in inoculated plots. In addition, we used indicator species analysis (indispecies package) to identify prokaryotic and fungal taxa that were significantly associated with Inoculated samples in at genera level, but none of the taxa were deemed as significantly associated. In the field, overall soil chemical properties were comparable among treatments (Table S7.4). Significant differences were found only for soil pH and available phosphate (P Olsen), and these occurred between seed mixtures, not between inoculation treatments within the same seed mixture. Specifically, soil pH was significantly higher in the EHR- than in the PHR- treatment (t = 3.212, p = 0.0375). For available phosphate, values were significantly higher in MC+ compared with both EHR- (t = 3.361, p = 0.0268) and PHR- (t = 3.871, p = 0.0080).

Functional groups

We observed no clear shift on the relative abundance of any prokaryotic or fungal functional guild (Figs. S7.3 & S7.4). However, for the field experiment, both control and inoculated field plots showed a gradual directional shift toward the donor community (Fig. S7.4), particularly for certain prokaryotic guilds involved in nitrification; notably aerobic ammonia oxidation and aerobic nitrite oxidation and for pathotrophic fungi. When comparing environments (greenhouse vs field), contrasts emerged. In the prokaryotic community, some guilds showed environment-specific enrichment: methylotrophy and methanol oxidation were nearly absent in the field but more prominent in greenhouse soils, whereas nitrification-related guilds exhibited the opposite trend. For fungal communities, responses were generally more consistent across environments. Guilds such as bryophyte parasites remained at very low relative abundances in both the greenhouse and field experiment, while dung saprotrophs and wood saprotrophs maintained relatively high abundances across all treatments. Small but consistent differences were visible for pathotrophs and leaf saprotrophs, which appeared slightly more abundant in field than in greenhouse soils.

7.4 Discussion

This study combined a controlled greenhouse experiment and a two-year field experiment to investigate whether whole-soil inoculation induces detectable shifts in microbial community composition and functional group structure during early stages of grassland conversion. Partly in line with our hypothesis, soil inoculation led to detectable shifts in microbial community composition during nine months in the greenhouse, but not during two years in the field. These contrasting outcomes suggest that agricultural soil conditions impose barriers to establishment that are absent in sterilised substrates under controlled conditions, consistent with our hypothesis.

7.4.1 Inoculation effects on microbial community composition

In the greenhouse experiment, containers receiving fresh donor soil developed prokaryotic and fungal communities most similar to the original donor soil, whereas sterilised donor soil produced distinct communities. Since both inocula shared (to a large extend) similar physical and chemical properties, this indicates that the observed community shifts were likely attributable to the living biota in the inoculum, rather than to abiotic differences between donor and field soil. The fact that inoculation treatment explained more variation in microbial community composition than seed mixture further underlines that inoculation was the primary driver of community assembly in the greenhouse. However, in contrast to our hypothesis, during the two years of field experiment, no clear inoculation signal emerged in microbial community composition regardless of seed mixture. This difference in response signal between controlled and field studies was to be expected based on findings from other studies that show that the effect size in controlled experimental settings is larger compared to field set-up (Azarbad & Junker, 2024; de Deyn et al., 2003, 2004; Forero et al., 2019). The absence of signal was mirrored in findings from Heinze et al. (2016), who similarly found that plant-soil feedback signals detected under controlled conditions were weakened or absent in the field. They attributed those slow responses to the greater complexity of biotic and abiotic interactions under natural conditions, such as the variability in soil moisture, light intensity and temperature. In addition to these general differences between controlled and field settings, several methodological factors specific to our field design may also have contributed to the limited inoculation effect. Specifically, only a thin inoculum layer was applied which was a deliberate choice to maximise practicality and minimise donor soil extraction (Gerrits et al., 2023). Han et al. (2022) showed that inoculation effects strengthen with layer thickness (3 and 5 cm versus 1 cm), so our approach may have introduced insufficient biomass to overcome

competitive resistance from the resident community. Scaling up layer thickness, however, would require more extensive excavation of semi-natural donor grasslands. A more practical alternative may be self-sourcing: farmers could develop and maintain herb-rich grassland donor pools specifically to supply inoculum for nearby fields. Soil inoculum from nearby locations (e.g. "local") could be more suitable for inoculation (Emam, 2016b; Han et al., 2022). It should be realized that the upscaling of the thickness of the layer also changes the nature of the treatment, as at 5 cm it borders on full soil translocation (Gerrits et al., 2023).

In addition, the level of prior disturbance may have been insufficient to promote establishment of introduced taxa. In one field inoculation study, topsoil removal enhanced inoculation success and there were clear developments of both soil and -unsown- plant community composition towards the donors (Wubs et al., 2016). In our field experiment, which was situated in a real farmers field, arable to grassland conversion apparently did not provide the same competitive release as complete topsoil removal (Geissen et al., 2013; Jaunatre et al., 2014). Another point is that two years after inoculation may simply not be long enough. Wubs et al. (2016) and Han et al. (2022) both found that inoculation effects became stronger when considering longer time periods, with significant community shifts emerging after six or more years. This creates a practical tension, as temporal grasslands in Dutch agriculture are typically maintained for only five years (RVO, code 266), meaning inoculation effects may fall outside the relevant management window for some farmers. For permanent grasslands, which persist longer than five years, the timeframe may be more compatible, but long-term experimental treatments followed by monitoring remains essential to determine whether investing in early-stage inoculation indeed translates into belowground community change on the longer term. In addition, functional consequences in terms of increased provisioning of ecosystem services and multifunctionality also may need to be included in such long-term monitoring (Guo et al., 2021).

Apart from the absence of an inoculation effect, sampling period significantly influenced microbial community composition in both control and inoculated plots. When taking both years together, sampling period explained the majority of variation in community composition (2.2% and 17.1% for prokaryotes and fungi, respectively), and communities in all plots shifted gradually towards the donor community over time regardless of inoculation treatment. The significant effect of seed mixture on microbial community composition (PERMANOVA; 4.8%, $p = 0.001$ for 16S and 4.3%, $p = 0.001$ for ITS2) suggests that plant community identity contributes to shaping belowground composition, in line with plant-microbe feedbacks reported elsewhere (Heinze et al., 2016). Together, these patterns are consistent with a gradual development of the belowground microbial

community during conversion from arable to grassland, driven at least in part by the developing plant community through root exudates and litter inputs. However, it remains unclear whether the temporal shift in microbial communities was primarily driven by plant community development, or whether it reflects autonomous successional processes within the soil community independent of vegetation. However, from our data alone, this cannot be resolved, since this would require vegetation-free control plots. It is also worth noting that the plant communities themselves did not necessarily shift towards the composition of the donor vegetation, specifically for the plots with monoculture rye grass, or the productive herb rich plots, meaning that the convergence of soil communities towards the donor may reflect broader arable-to-grassland successional dynamics rather than plant-mediated steering towards a specific target community.

7.4.2 Indicator taxa of inoculation success

In the greenhouse, 78 prokaryotic and 94 fungal ASVs were uniquely introduced by inoculating the donor soil. Indicator species analysis identified three prokaryotic genera (Blastococcus, Flavihumibacter, and an unknown genus) and ten fungal genera (Clonostachys, Staphylotrichum, Clohesyomyces, Slopeiomyces, Neopyrenochaeta, Ophiosphaerella, Hirsutella, Gaeumannomycella, and Pyrenochaeta) as significantly associated with the inoculated treatment, and present in the donor community. These taxa may represent the key drivers of the community shift observed in the greenhouse. In the field, 143 prokaryotic and 43 fungal ASVs were uniquely present in inoculated plots, while they were not present in the control plots, suggesting that they have been introduced with the donor soil. However, none of these taxa showed significantly increased in abundance over one or two years, and indicator species analysis found no taxa significantly associated with the inoculated treatment at genus level. The introduction of taxa therefore did not guarantee their proliferation. Microbes may establish at low densities but fail to expand, possibly due to environmental filtering, competition from the resident community, or insufficient time to accumulate. This distinction between presence and abundance is important: detecting introduced taxa by presence-absence alone may overestimate establishment success, at least at the short term. Unravelling possible causes of such time lags will require further studies.

7.4.3 Functional group responses

Inoculation did not lead to significant shifts in microbial functional group composition neither in the greenhouse nor the field. In the greenhouse, there were non-significant

trends of a directional shift in functional community structure towards the donor (Fig. S7.3). In the field, functional group composition changed over time, again possibly driven by plant community development or another autonomous temporal effect, but inoculation itself did not accelerate this. This raises the question of how long functional recovery might take: while plant communities can be steered relatively quickly through seeding, the timescales for corresponding shifts in soil functionality remain poorly characterised (although see Resch et al., 2021). Some indirect evidence comes from non-grassland systems: in arable soils, repeated compost amendment over more than five years displaced indigenous methane-cycling community members, though this compositional shift was not identified as the primary driver of increased CH_4 uptake potential. The compost appeared to act more as a vector for introducing specific functional taxa than as a direct modifier of functioning (Van Den Bergh et al., 2025). This raises a broader question relevant to inoculation-based restoration: whether functional consequences of community change depend not just on whether new taxa are introduced, but on the abundance and frequency of inoculum application, the relative abundance of functionally relevant taxa within the inoculum, and whether the taxa being introduced hold unique functional roles. These questions remain unresolved and underscore the need for long-term experimental approaches that track both community composition and functional outcomes simultaneously.

7.4.4 Implications for practice and outlook

The findings in our combined greenhouse and field study have direct implications for the use of whole-soil inoculation as a tool in agricultural grassland restoration. Under controlled greenhouse conditions, inoculation with donor soil successfully steered both prokaryotic and fungal communities towards the donor community, with living biota as possible key drivers of this shift. Indicator taxa associated with inoculated treatments (including fungal genera such as Clonostachys, Hirsutella, and Ophiosphaerella, and prokaryotic genera including Blastococcus and Flavihumibacter) represent candidate organisms for future monitoring of inoculation success. In contrast, it is likely that the agricultural context creates specific constraints that field inoculation studies in semi-natural systems do not always face: soil legacies from fertilisation, and the need for visible returns within a timeframe meaningful to farmers (Kleijn et al., 2019). Commercial microbial inocula, single-strain or synthetic consortia, are already widely used in agriculture (French et al., 2017; O'Callaghan et al., 2022), but typically produce short-lived effects that rarely persist beyond a single season (Emam, 2016). Whole-soil inoculation, by introducing a heterogeneous community, has the potential for more durable effects,

but our results suggest it does not act fast enough under realistic agricultural conditions to serve a short-term management goal. This does not preclude its long-term potential, as inoculation effects are known to become more pronounced over time (Han et al., 2022), but it does highlight the need for complementary measures, such as topsoil management to reduce competitive resistance The possible role of other management actions, such as larger inoculum quantities, need further studies The seed mixture used alongside inoculation may be an important modifying factor. In Dutch agricultural grasslands, productive herb-rich mixtures (high legume content) and extensive herb-rich mixtures (high plant diversity) differ substantially in how they structure the plant community, which in turn could shape belowground assemblages, although this could also take a considerable amount of time depending on the management (Hannula et al., 2021; Van Eekeren et al., 2024). Whether certain mixtures create a more favourable soil environment for inoculant establishment requires further investigation, including longer monitoring periods, integration with topsoil management practices that reduce competitive resistance and/or the possible use of larger inoculum quantities where feasible. Establishing donor grassland pools within farms might offer an innovative approach that addresses both the practical and ecological constraints identified here.

7.5 Data availability statement

Data available from the Zenodo data repository: https://doi.org/10.5281/zenodo.19454723

Chapter appendix

7.A Supplementary figures

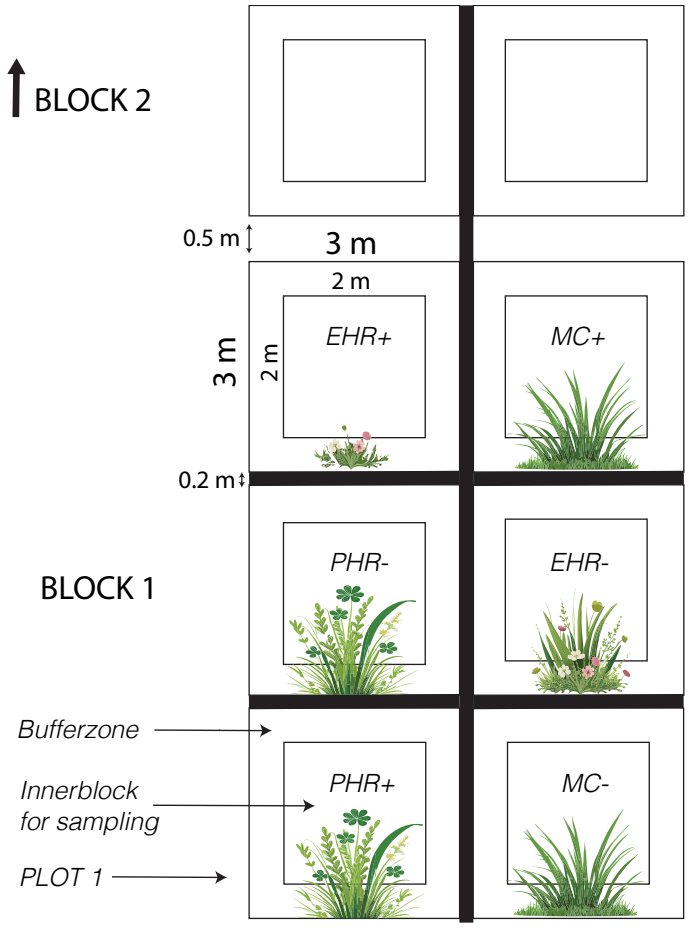


Figure S7.1: Experimental field set-up

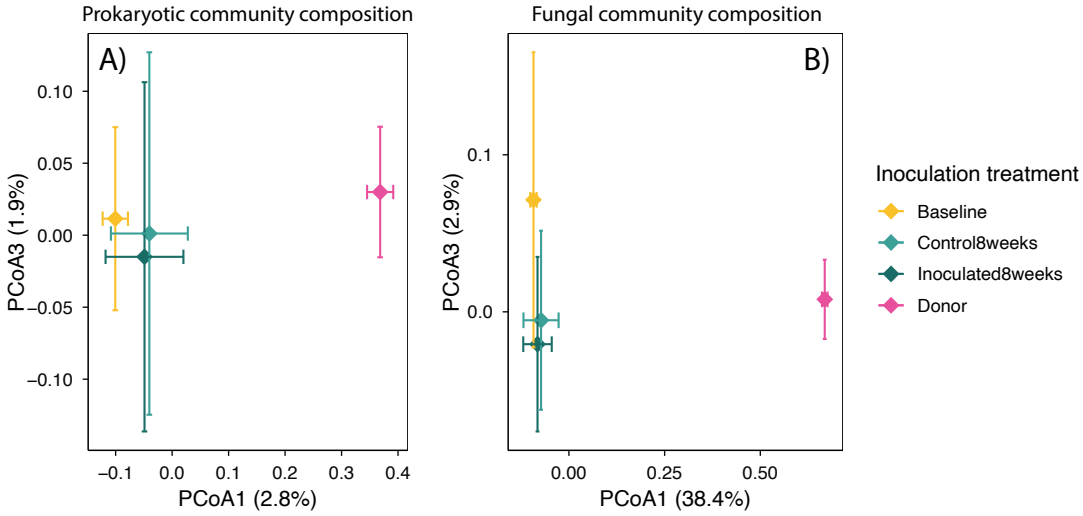


Figure S7.2: Principle Coordinate Analysis (PCoA) of A) 16s community, based on OTU's and B) ITS2 community, based on OTU's, grouped per treatment type (Baseline = Receiving site before inoculation, Control8weeks = non-inoculated plots after 8 weeks of inoculation, Inoculated8weeks = inoculated plots after 8 weeks of inoculation.

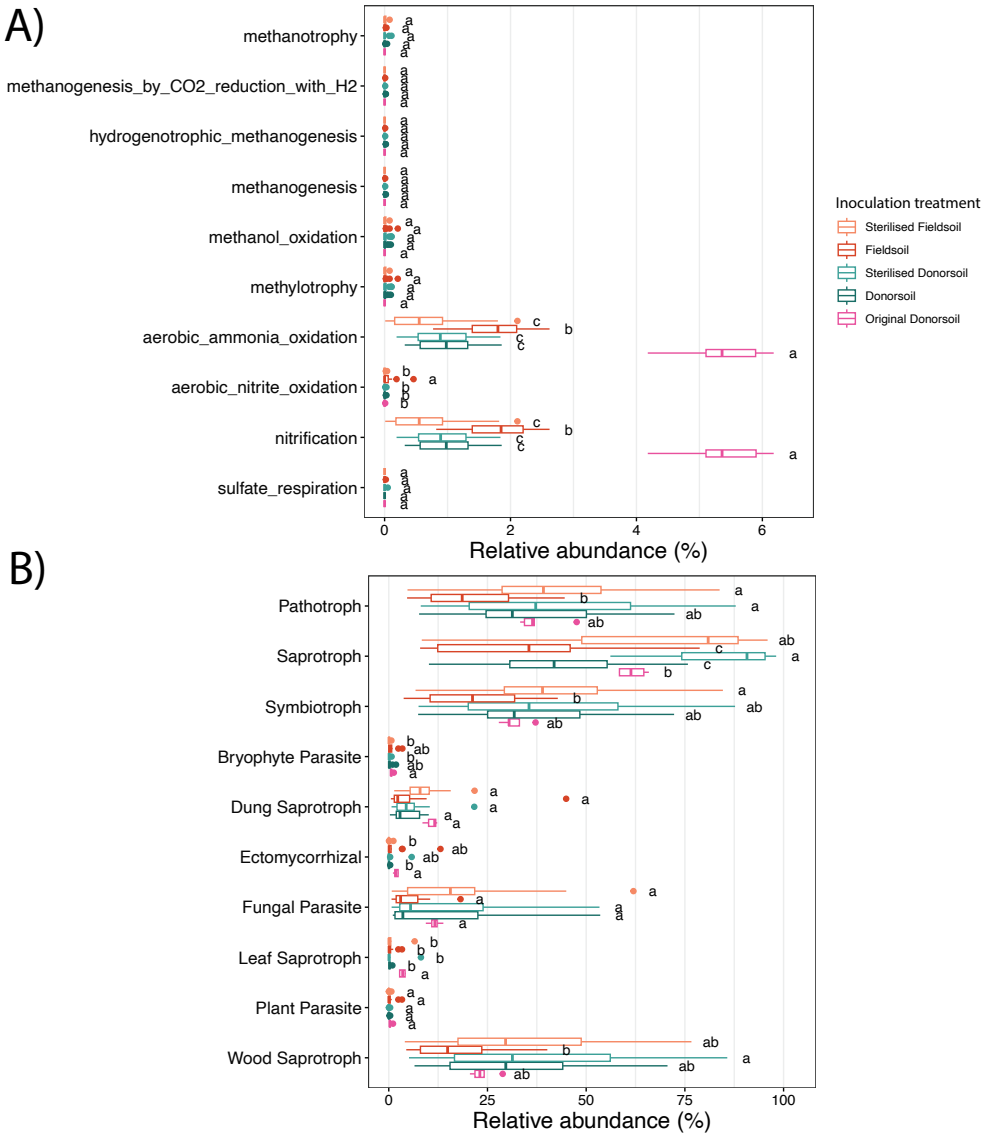


Figure S7.3: Results from FARPOTAX and FUNGuild analysis for 16S (A) and ITS (B) for the greenhouse experiment. Significance letters (a,b,c,d) show signifcant differences ($p<0.05$) between groups based on relative abundances (ASVs) of 16S and ITS2 genera.

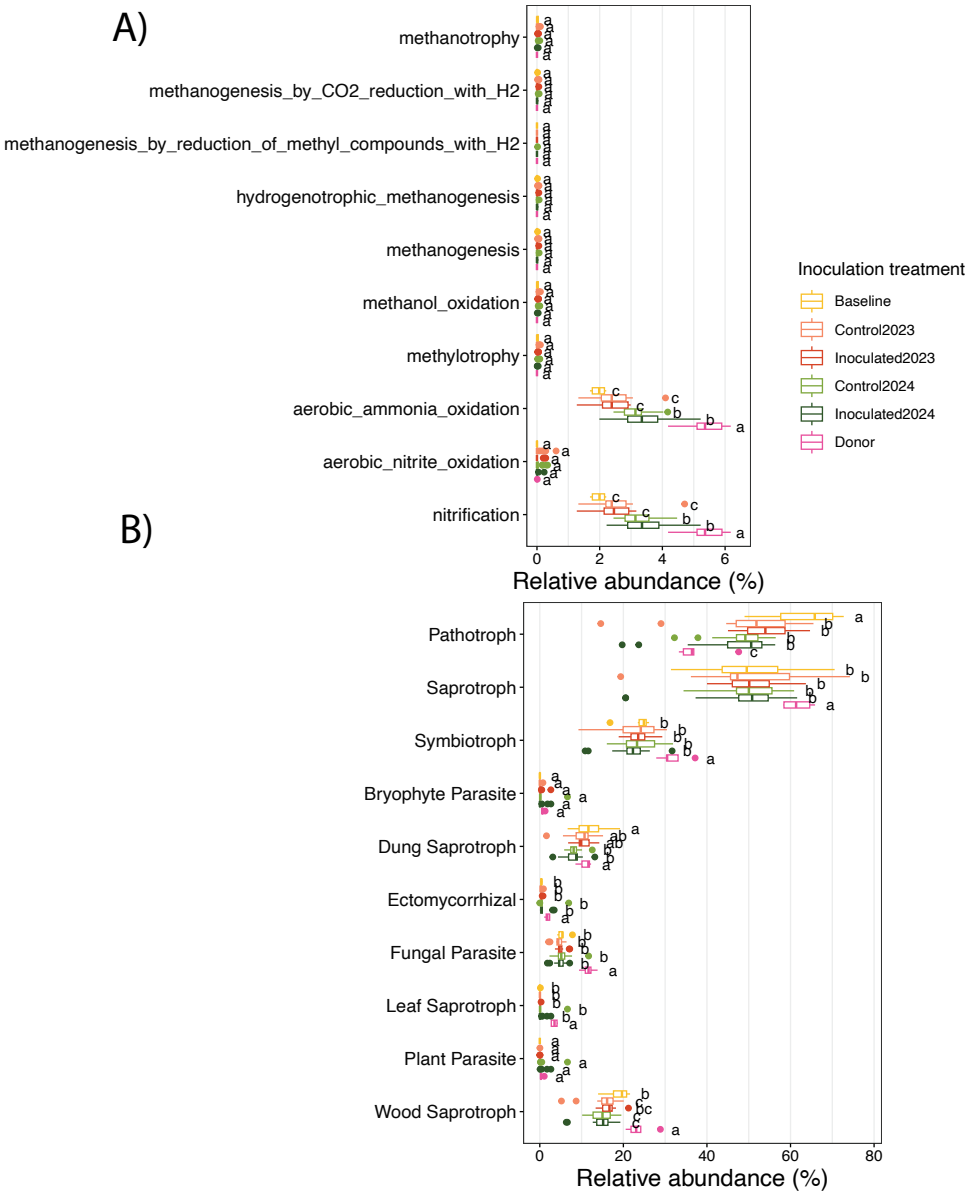


Figure S7.4: Results from FARPOTAX and FUNGuild analysis for 16S (A) and ITS (B) for the field experiment. Significance letters (a,b,c,d) show signifcant differences ($p<0.05$) between groups based on relative abundances (ASVs) of 16S and ITS2 genera.

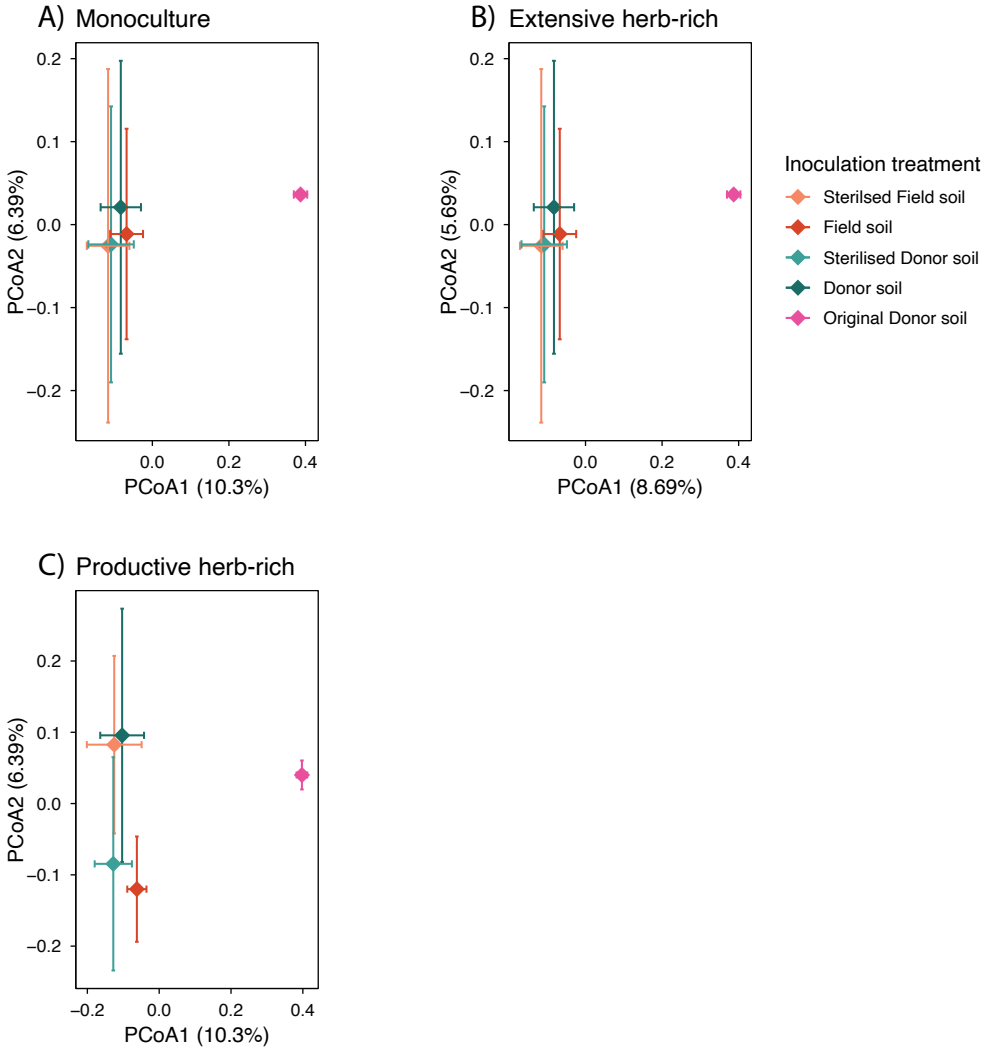


Figure S7.5: Principal Coordinates Analysis (PCoA) of bacterial community composition (16S) based on Bray-Curtis dissimilarity, shown separately for each seed mixture treatment: (A) Monoculture (MC), (B) Extensive herb-rich (EHR), and (C) Productive herb-rich (PHR). Points represent group centroids and error bars indicate standard deviation. The percentage of variance explained by each axis is shown in parentheses. Five inoculation treatments are compared: (1) field soil; non-sterilised soil from the recipient field, representing the resident microbial community; (2) sterilised field soil; sterilised recipient field soil, serving as an abiotic control without a living microbial community; (3) donor soil non-sterilised soil from the species-rich donor grassland, containing the microbial inoculum intended for inoculation; (4) sterilised donor soil; sterilised donor soil, controlling for the chemical and physical properties of the donor soil without its microbial community; and (5) original donor soil; a reference sample taken directly from the donor grassland, representing the target community state.

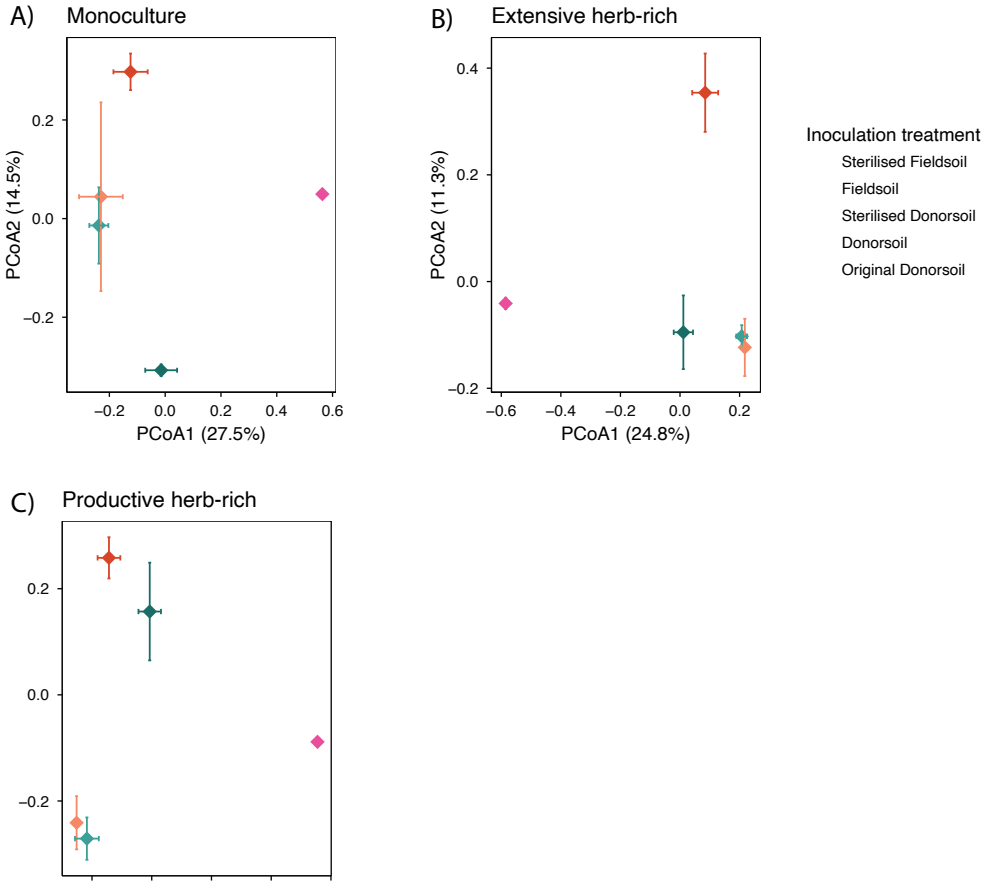


Figure S7.6: Principal Coordinates Analysis (PCoA) of fungal community composition (ITS2) based on Bray-Curtis dissimilarity, shown separately for each seed mixture treatment: (A) Monoculture (MC), (B) Extensive herb-rich (EHR), and (C) Productive herb-rich (PHR). Points represent group centroids and error bars indicate standard deviation. The percentage of variance explained by each axis is shown in parentheses. Five inoculation treatments are compared: (1) field soil; non-sterilised soil from the recipient field, representing the resident microbial community; (2) sterilised field soil; sterilised recipient field soil, serving as an abiotic control without a living microbial community; (3) donor soil non-sterilised soil from the species-rich donor grassland, containing the microbial inoculum intended for inoculation; (4) sterilised donor soil; sterilised donor soil, controlling for the chemical and physical properties of the donor soil without its microbial community; and (5) original donor soil; a reference sample taken directly from the donor grassland, representing the target community state.

7.B Supplementary tables

Table S7.1: Results from an irrigation event in 2023, showing soil moisture content and the volume of water received per treatment plot. Soil moisture content (%) was measured one day after irrigation. The volume of irrigation water (mL) received per plot was collected using rain water gauges placed within each plot. Treatments correspond to the three seed mixture types; Monoculture (MC), Extensive herb-rich (EHR), and Productive herb-rich (PHR), each applied either with (+) or without (−) whole-soil inoculation from a donor grassland. Values are plot means; overall averages and standard deviations are reported in the bottom row. The similar moisture contents and water volumes across treatments confirm that irrigation was applied uniformly, with no systematic differences between treatment groups (overall mean moisture: 16.80 $\pm$ 0.85%; overall mean irrigation volume: 99.69 $\pm$ 4.77 mL).

Treatment	Moisture content (%)	mL added through irrigation
MC−	16.09	96.83
MC+	17.90	106.83
EHR−	17.07	96.50
EHR+	17.49	94.33
PHR−	16.59	100.00
PHR+	15.65	103.67
Average	16.80	99.69
SD	0.85	4.77

Table S7.2: Results of pairwise PERMANOVA testing differences in bacterial community composition (16S) between sampling time points and treatment groups from the field experiment, based on Bray-Curtis dissimilarity. Groups represent combinations of inoculation treatment (Inoculated vs. Control) and sampling moment (8 weeks after inoculation, 2023, 2024), alongside a pre-treatment Baseline and the original Donor soil as a reference. Reported statistics include the F-statistic (F), the proportion of variance explained (R^2), the unadjusted p-value (p.value), and the Benjamini-Hochberg adjusted p-value (p.adjusted). Significance is indicated as: * p < 0.05, ** p < 0.01; empty cells indicate non-significant comparisons (ns).

Groups	F	R^2	p.value	p.adjusted	Significance
Inoculated 2023 vs Control 2023	1.01	0.03	0.331	0.356	ns
Inoculated 2023 vs Inoculated 2024	1.09	0.03	0.018	0.023	*
Inoculated 2023 vs Control 2024	1.07	0.03	0.044	0.051	ns
Inoculated 2023 vs Inoculated 8 weeks	1.10	0.03	0.004	0.007	**
Inoculated 2023 vs Control 8 weeks	1.08	0.03	0.012	0.018	*
Inoculated 2023 vs Baseline	1.08	0.05	0.018	0.023	*
Inoculated 2023 vs Donor	2.20	0.09	0.001	0.002	**
Control 2023 vs Inoculated 2024	1.15	0.03	0.001	0.002	**
Control 2023 vs Control 2024	1.11	0.03	0.006	0.009	**
Control 2023 vs Inoculated 8 weeks	1.15	0.03	0.001	0.002	**

Groups	F	R^2	p.value	p.adjusted	Significance
Control 2023 vs Control 8 weeks	1.14	0.03	0.001	0.002	**
Control 2023 vs Baseline	1.10	0.05	0.013	0.018	*
Control 2023 vs Donor	2.23	0.09	0.001	0.002	**
Inoculated 2024 vs Control 2024	1.01	0.03	0.364	0.377	ns
Inoculated 2024 vs Inoculated 8 weeks	1.23	0.03	0.001	0.002	**
Inoculated 2024 vs Control 8 weeks	1.22	0.03	0.001	0.002	**
Inoculated 2024 vs Baseline	1.24	0.05	0.001	0.002	**
Inoculated 2024 vs Donor	2.32	0.10	0.001	0.002	**
Control 2024 vs Inoculated 8 weeks	1.27	0.04	0.001	0.002	**
Control 2024 vs Control 8 weeks	1.21	0.04	0.001	0.002	**
Control 2024 vs Baseline	1.17	0.05	0.001	0.002	**
Control 2024 vs Donor	2.17	0.09	0.001	0.002	**
Inoculated 8 weeks vs Control 8 weeks	0.89	0.03	1.000	1.000	ns
Inoculated 8 weeks vs Baseline	1.06	0.05	0.062	0.069	ns
Inoculated 8 weeks vs Donor	2.21	0.09	0.001	0.002	**
Control 8 weeks vs Baseline	1.07	0.05	0.028	0.034	*
Control 8 weeks vs Donor	2.15	0.09	0.001	0.002	**
Baseline vs Donor	2.15	0.18	0.002	0.004	**

Table S7.3: Results of pairwise PERMANOVA testing differences in fungal community composition (ITS2) between sampling time points and treatment groups from the field experiment, based on Bray-Curtis dissimilarity. Groups represent combinations of inoculation treatment (Inoculated vs. Control) and sampling moment (8 weeks after inoculation, 2023, 2024), alongside a pre-treatment Baseline and the original Donor soil as a reference. Reported statistics include the F-statistic (F), the proportion of variance explained (R^2), the unadjusted p-value (p.value), and the Benjamini-Hochberg adjusted p-value (p.adjusted). Significance is indicated as: * p < 0.05, ** p < 0.01; empty cells indicate non-significant comparisons (ns).

Groups	F	R^2	p.value	p.adjusted	Significance
Inoculated 2023 vs Control 2023	0.62	0.02	0.98	0.98	
Inoculated 2023 vs Inoculated 2024	2.76	0.08	0.001	0.001	**
Inoculated 2023 vs Control 2024	2.68	0.07	0.001	0.001	**
Inoculated 2023 vs Inoculated 8 weeks	7.67	0.19	0.001	0.001	**
Inoculated 2023 vs Control 8 weeks	7.48	0.19	0.001	0.001	**
Inoculated 2023 vs Baseline	2.91	0.13	0.001	0.001	**
Inoculated 2023 vs Donor	20.96	0.53	0.001	0.001	**

Groups	F	R^2	p.value	p.adjusted	Significance
Control 2023 vs Inoculated 2024	2.07	0.06	0.02	0.02	*
Control 2023 vs Control 2024	2.41	0.07	0.001	0.001	**
Control 2023 vs Inoculated 8 weeks	7.15	0.18	0.001	0.001	**
Control 2023 vs Control 8 weeks	7.20	0.18	0.001	0.001	**
Control 2023 vs Baseline	2.46	0.11	0.002	0.003	**
Control 2023 vs Donor	15.39	0.46	0.002	0.003	**
Inoculated 2024 vs Control 2024	0.89	0.03	0.53	0.57	
Inoculated 2024 vs Inoculated 8 weeks	12.62	0.27	0.001	0.001	**
Inoculated 2024 vs Control 8 weeks	12.01	0.26	0.001	0.001	**
Inoculated 2024 vs Baseline	3.91	0.15	0.001	0.001	**
Inoculated 2024 vs Donor	18.63	0.48	0.001	0.001	**
Control 2024 vs Inoculated 8 weeks	14.21	0.29	0.001	0.001	**
Control 2024 vs Control 8 weeks	13.56	0.28	0.001	0.001	**
Control 2024 vs Baseline	4.63	0.17	0.001	0.001	**
Control 2024 vs Donor	23.42	0.52	0.001	0.001	**
Inoculated 8 weeks vs Control 8 weeks	0.80	0.02	0.73	0.76	
Inoculated 8 weeks vs Baseline	2.25	0.09	0.009	0.01	*
Inoculated 8 weeks vs Donor	32.52	0.60	0.001	0.001	**
Control 8 weeks vs Baseline	2.21	0.09	0.01	0.01	*
Control 8 weeks vs Donor	29.99	0.58	0.001	0.001	**
Baseline vs Donor	16.92	0.65	0.003	0.004	**

Table S7.4: Results of chemical soil analysis for the field experiment sampled in 2024, shown per treatment combination. Treatments correspond to the three seed mixture types; Monoculture (MC), Extensive herb-rich (EHR), and Productive herb-rich (PHR), each applied either with (+) or without (−) whole-soil inoculation from a donor grassland. Measured variables include Organic Matter content (OM, %), bulk density (BD, g/cm^3), gravimetric moisture content (%), soil acidity (pH), plant-available phosphorus (Olsen P, kg/ha), total nitrogen (N, %), total carbon (C, %), and soil organic carbon (SOC, %). Values are presented as mean ± standard deviation. Different lowercase letters within a column indicate statistically significant differences between treatments (post-hoc comparison, $p < 0.05$); treatments sharing the same letter do not differ significantly.

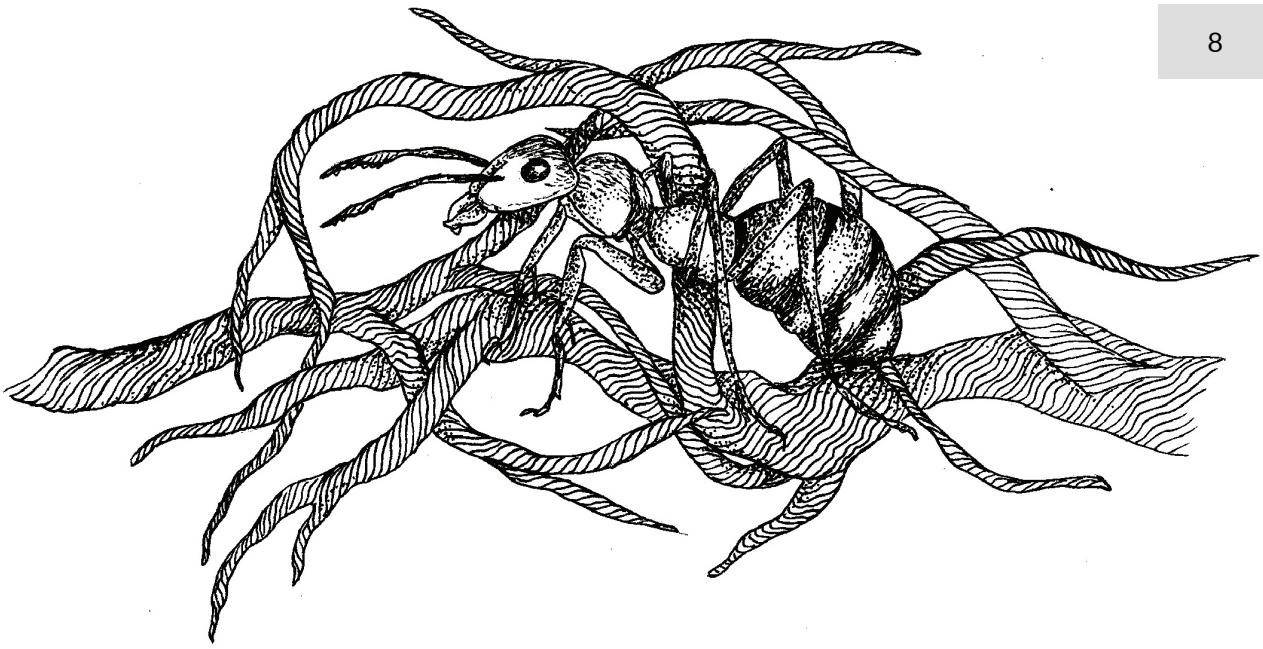
Type	OM (%)	BD	Moisture (%)	Acidity pH	Olsen P (kg/ha)	N (%)	C (%)	SOC (%)
MC+	4.23 ± 0.32	1.37 ± 0.05	25.22 ± 1.21	6.08 ± 0.08^{a}	138.57 ± 12.95^{a}	0.17 ± 0.01	2.17 ± 0.14	2.16 ± 0.12
MC−	4.09 ± 0.19	1.35 ± 0.04	24.29 ± 0.13	5.94 ± 0.12^{a}	122.19 ± 6.55^{ab}	0.16 ± 0.01	2.04 ± 0.08	2.05 ± 0.07
EHR+	4.19 ± 0.27	1.34 ± 0.03	24.16 ± 0.73	5.99 ± 0.05^{a}	123.28 ± 9.98^{ab}	0.17 ± 0.01	2.10 ± 0.17	2.11 ± 0.17
EHR−	4.18 ± 0.19	1.33 ± 0.06	23.77 ± 0.61	6.02 ± 0.11^{b}	120.56 ± 8.97^{b}	0.16 ± 0.01	2.07 ± 0.14	2.06 ± 0.16
PHR+	4.13 ± 0.13	1.38 ± 0.05	23.57 ± 0.66	6.01 ± 0.12^{a}	131.27 ± 9.38^{ab}	0.16 ± 0.01	2.06 ± 0.05	2.02 ± 0.04
PHR−	4.14 ± 0.05	1.36 ± 0.05	23.15 ± 0.59	5.81 ± 0.17^{c}	117.82 ± 6.19^{b}	0.16 ± 0.00	2.06 ± 0.04	2.08 ± 0.05



Photograph taken at the author's family farm in Zeeland, the Netherlands.

8

Synthesis



Agricultural intensification and its limits

How do soils influence the transition towards sustainable agricultural systems? This question has occupied soil ecologists and agronomists for years, yet how to measure and interpret such transitions under real field conditions remains poorly understood. The urgency of understanding how soils respond to this transition is clear: the dominant high-input, high-output model of agricultural production generates substantial environmental externalities such as nitrogen pollution, biodiversity loss, and soil degradation, which increasingly undermine the ecological systems on which agriculture itself depends (Erisman et al., 2013; Kopittke et al., 2019; Pretty, 2018; Tsiafouli et al., 2015). In countries such as the Netherlands, with highly industrialised agricultural systems, the challenge is no longer whether to reduce these external impacts, but how to do so while maintaining sufficient food production (MacLaren et al., 2022; Staniszewski et al., 2023). Agricultural extensification offers a promising pathway to sustainability: by reducing dependence on external inputs and relying on internally regulated soil processes, extensification aims to decouple productivity from environmental harm (van Grinsven et al., 2015). Central to this transition is the soil microbial community, which drives important soil processes such as decomposition, respiration and nutrient mineralisation (Bardgett & van der Putten, 2014) that can alleviate the dependence of these systems on external synthetic inputs (Bommarco et al., 2013). Yet the capacity of soils to support these processes is hampered by the legacy of decades of agricultural intensification that is stored in the physical, chemical, and biological properties of soils (van der Putten et al., 2013). These legacy effects in the soil that result from former land use may constrain or enable the functional transition that extensification requires.

This thesis was motivated by three interlinked questions that together frame extensification as a soil transition (Fig. 8.1): (1) how do soil properties, microbial communities and functioning vary along a gradient from intensive to extensive management, (2) to what extent can these differences be detected and monitored using novel indicators, and (3) are there ways to actively accelerate this transition. At the core of the thesis lies a management gradient of permanent grasslands in the Ooijpolder, which formed the foundation for studying how soil properties, microbial communities and functioning vary under long-term differences in land management intensity (Chapters 2 and 3). Building on this, the thesis explored whether microbial volatile organic compounds (mVOCs) (metabolites produced by soil-borne bacteria and fungi) that reflect microbial community structure and activity could serve as a novel, non-destructive proxy for tracking soil community change along management gradients (Chapters 4, 5 and 6). Finally, a soil inoculation experiment tested whether the transition towards more extensive soil communities could be actively

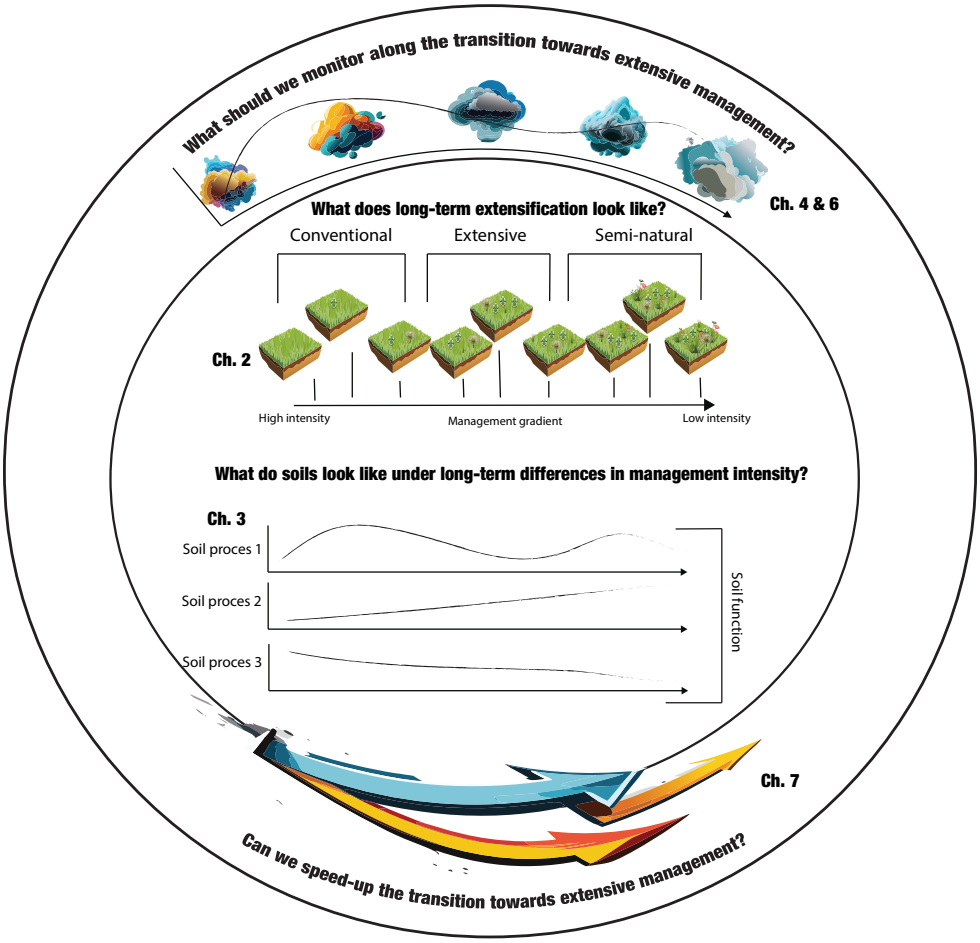


Figure 8.1: Conceptual overview of the thesis. The three overarching research questions are arranged as concentric rings: what long-term extensification looks like across a management gradient (Chapters 2 and 3), what can be monitored along this transition (Chapters 4 and 6), and whether the transition can be actively steered (Chapter 7). The central panel illustrates the core finding of Chapter 3: that soil processes respond to management intensity with different sensitivities and trajectories, jointly giving rise to soil functioning.

steered by introducing soil biota from extensively managed reference sites (Chapter 7). Together, these three lines of inquiry are addressed in this Discussion and Synthesis.

Whether gradients work better than categories: conceptualising extensification as a trajectory

Agricultural management decisions are shaped by local environmental conditions, economic constraints and farmers' preferences. Every farmer makes decisions based on

the own personal situation, which creates farming systems with unique combinations of practices and outcomes (Köbrich et al., 2003). Often, this diversity has been simplified by grouping farms into discrete categories, for example land-use types, under the assumption that systems with broadly comparable management would respond similarly and could therefore receive comparable recommendations (Köbrich et al., 2003; Lesschen et al., 2005). While this approach has practical advantages, it risks obscuring ecological variability that arises within categories: farms classified under the same land-use type can differ substantially in their actual management practices, which may translate into considerable variation in ecological properties (Armengot et al., 2011; van Rijssel et al., 2025). The results of this thesis partially support this notion of variability within land-use types. The grasslands in this study were initially selected based on a priori categories ("conventional", "extensive" and "semi-natural") but Chapter 2 demonstrated that management intensity was more coherently described by a continuous gradient derived from farmer questionnaires, capturing the combined influence of fertilisation regime, grazing intensity and plant diversity. To assess this gradient independently of the response variables themselves, we validated it against an objective remote-sensing measure of land use intensity (S2REP), rather than selecting between gradient and categorical representations based on which better fitted the soil data. This approach avoided circularity in the comparison. However, with the full dataset now available across chapters, it is possible to revisit this comparison by an additional analysis comparing the explanatory power of the gradient against categorical classifications for the soil parameters measured in Chapters 3 and 4 (Box 1, Table 1).

Box 1. Model comparison between categorical and gradient representations of management

To evaluate whether soil responses were better explained by discrete management categories or by a continuous management gradient, I compared mixed-effects models including either management Type (categorical factor) or the management gradient (continuous predictor) as fixed effects. For each soil variable, two models with identical random-effects structure were fitted:

1. response $\sim$ gradient + (1 | Field)
2. response $\sim$ Type + (1 | Field)

Field identity was included as a random intercept to account for spatial dependence among samples collected within the same site. Models were fitted using linear mixed-effects models (lmer, package `lme4`) for approximately normally distributed responses and using `glmmTMB` with a Student-t error distribution where residual diagnostics indicated deviations from normality. Model support was compared using Akaike's Information Criterion (AIC) based on maximum likelihood estimation. Differences in model fit were interpreted using ΔAIC values, with differences < 2 considered to indicate equivalent support. Likelihood ratio tests were additionally performed to assess statistical differences between models. Residual diagnostics were evaluated using the `DHARMa` package to verify model assumptions.
For variables showing evidence that both predictors contributed to variation, an additional model including both fixed effects was fitted: response $\sim$ gradient + Type + (1 | Field). This allowed assessment of whether categorical management effects explained variance beyond the continuous gradient.

Table 1

Variable	Best model (Type vs gradient)	ΔAIC (Type – Gradient)	ANOVA p-value	Interpretation
Respiration	Similar	$\sim$0–1	0.39	weak / gradual
Decomposition	Similar	–0.6	0.107	gradual continuum
Enzymes	Type	–2.2	0.041*	partial clustering
ITS2 (fungi)	Type	–20.4	$<$0.001*	strong clustering
16S (bacteria)	Type	–2.5	0.035*	moderate clustering
pH	Both	–	gradient ns alone; both sig when combined	hierarchical structure
Total N	Similar	+1.1	0.336	gradual
SOC	Gradient	+2.1	1.000	gradual
Bulk density	Gradient	+2.1	1.000	gradual
mVOCs (pcoa1)	Type	–3.1	0.024*	categorical signal present

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For many variables, the continuous gradient and categorical classifications ("Type") provided comparable explanatory power as shown by the relatively minor differences in model support, indicating that the observed variation was equally well explained by categorising management into types as well as identifying them based on a gradient of measured management characteristics. The equivalence implies that both representations are conceptually interchangeable. However, one possible advantages of the gradient, is that it can capture additional within-type variation that categorical models structurally cannot represent. Most soil processes visualised along the management gradient also did not show clear clustering (Fig. 3.5 Chapter 3). In such cases, a continuous gradient may more accurately reflect the underlying ecological variation, as it does not impose discrete boundaries on management decisions that are inherently continuous (Lesschen et al., 2005).

At the same time, several variables showed stronger support for categorical representations, including microbial community composition (16S and ITS2) and mVOC profiles, indicating that discrete clustering can emerge for specific biological components even when overall system organisation follows a continuous gradient. This could be explained by the fact that some variables (such as fungal communities) may shift abruptly when certain management thresholds are crossed (Fierer, 2017; Oehl et al., 2010). Soil pH further illustrated this hierarchical structure, with evidence for both categorical differences and continuous variation operating simultaneously. However, pH did not follow management categories but instead displayed a bimodal distribution across fields (Chapter 3, Fig. 3.4), with values clustering around higher (>7) and lower (<6) ranges irrespective of management classification. This phenomenon is likely driven by the individual decision of the farmer irrespective of land-use type regarding soil liming, which is a common practice to increase pH for the benefit of plant production. The observation in pH distribution illustrates how key abiotic variables may not be captured by categorised management practices, thereby influencing biological and functional responses in ways that obscure simple category-based interpretations (Lauber et al., 2008).

A complicating but important feature of considering management as a gradient is that the fields on that gradient may vary in history and historical legacies. The extensively and semi-naturally managed grasslands sampled here were all managed consistently for at least ten years, but they likely started from different points of management intensity and converted at different moments in time and with different intensities of extensification (Fig. 8.2). These differences in history could influence their current and future position on the management gradient (Briar et al., 2007; Louisson et al., 2023; Schrama et al., 2018; van Rijssel et al., 2022; Verbruggen et al., 2010). These differences in starting point, du-

ration and pace of transition were not captured in this study, as the research design focused on current management state rather than management history. Yet despite this hidden variation in trajectory, three soil parameters consistently correlated with the current management gradient: soil organic carbon, litter decomposition rate and fungal community composition (Chapter 3). The coherence of these responses across fields with presumably different histories suggests that these variables may be robust indicators in our study area, reflecting the current extensification status of a soil regardless of its history. This has practical implications: if SOC, decomposition and fungal community composition reliably reflect current management state, they may also serve as indicators for monitoring the progress of ongoing transitions toward extensification, and potentially as predictors of a soil's transition phase for the fields measured.

Conceptualising extensification as a trajectory also aligns more closely with how management changes occur in practice. Farmers rarely make an instantaneous transition between predefined regimes; instead, they typically adjust practices incrementally in response to evolving goals, constraints and opportunities. Exploratory interviews with farmers conducted in the context of this research (Chapter 5) suggested that they are often more interested in whether soils are changing following gradual adjustments than in comparisons between predefined categories. This perspective reinforces the relevance of gradient-based approaches for both ecological interpretation and practical decision-making. However, policy frameworks continue to rely largely on categorical classifications and certification such as "Stichting Keur Alternatief voortgebrachte Landbouwproducten" (SKAL) for organic farming systems (Skal Biocontrole, 2021). My study results strongly suggest that gradient-based approaches could offer greater flexibility by accommodating continuous variation and overlapping system characteristics, which may be particularly valuable for understanding ecological processes during transitions. Land-use types without certification, such as "conventional" are more difficult to be defined, because of the lack of official criteria (Sumberg & Giller, 2022). Emerging concepts such as regenerative agriculture may further complicate categorical definitions, as they emphasise sets of principles rather than prescriptive practices, potentially leading to substantial heterogeneity within categories (Newton et al., 2020; Schreefel et al., 2020). In this context, gradient-based frameworks may offer a more tractable basis for understanding ecological responses during transitions, precisely because they do not depend on rigorous categorical boundaries.

Overall, the results of this thesis indicate that agricultural extensification is better conceptualised as movement along a multidimensional management gradient than as a shift between discrete categories. This perspective provides a more realistic representation of

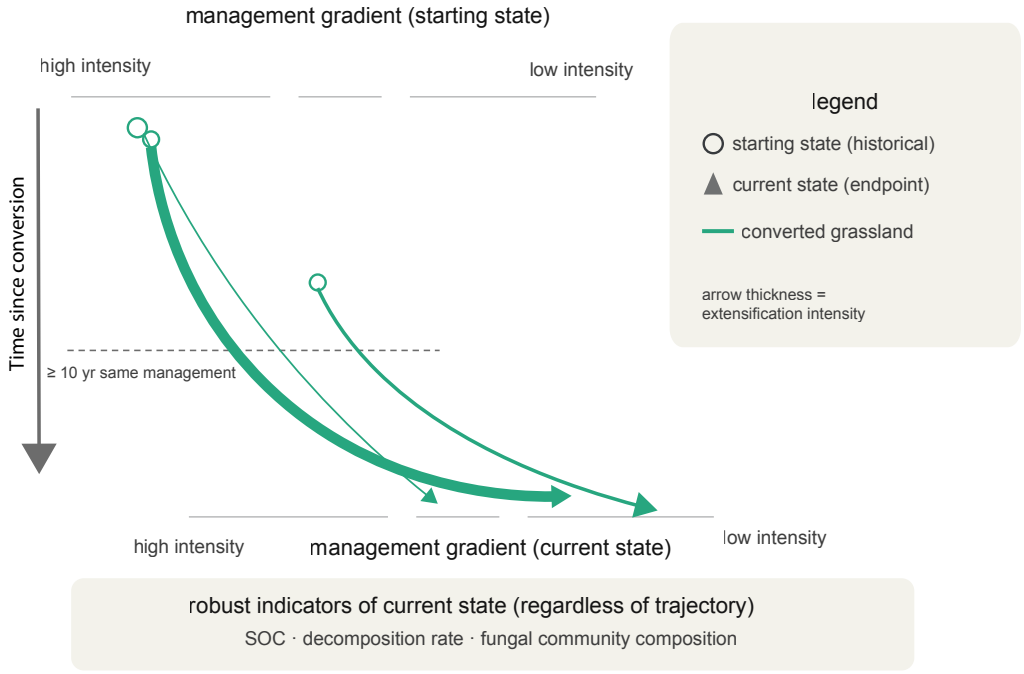


Figure 8.2: Conceptual diagram illustrating that fields occupying the same management gradient may have arrived there through different trajectories. Open circles represent the historical starting state of each field; filled triangles represent their current position on the management gradient. Arrow thickness indicates degree of extensification applied during conversion. Despite differences in starting point, duration and pace of transition, SOC, decomposition rate and fungal community composition emerged as robust indicators that consistently reflect current management state regardless of trajectory.

both farming practice and soil ecological responses, and it establishes the foundation for examining how soil systems may be reorganised along such trajectories. The following sections therefore focus on the mechanisms underlying soil reorganisation along a management gradient, and how these mechanisms influence soil functioning, predictability and management interventions. Mechanisms of soil reorganisation: fungal-mediated factors and abiotic conditioning by pH In this thesis, having established that extensification is more accurately conceptualised as a trajectory along a management gradient, the next question is what drives the reorganisation of soils along this gradient. In the present synthesis, the term soil reorganisation is used to describe the concerted, but not necessarily uniform, changes in soil biological communities, physicochemical properties and func-

tional processes that occur in response to differences in management intensity (Bloor et al., 2021; Creamer et al., 2016; Postma-Blaauw et al., 2012), as demonstrated in Chapters 3, 4 and 7). Concerted means that multiple soil properties are simultaneously exposed to the same external drivers, for example plant diversity, manure inputs and grazing intensity, which are known to jointly influence soil biological communities, alter soil chemistry, and shape functional processes in grasslands (Bardgett & van der Putten, 2014; Wardle et al., 2004). This does not imply a single uniform trajectory for each soil property, but rather different components respond with distinct sensitivities and different time scales. These types of responses are exemplified by the results of Chapters 3 and 7. In my thesis research, two soil properties emerged as consistently and strongly associated with this reorganisation along the management gradient: fungal community dynamics, and soil pH.

Fungal-associated axis of soil reorganisation

In Chapter 3, fungal community composition was significantly correlated with both soil organic carbon and litter decomposition rate, and model performance for decomposition improved when fungal community composition was included, though this effect remained marginal ($p = 0.08$). Similar patterns emerged in Chapter 4, where fungal community composition was identified as a key factor associated with differences in mVOC profiles along the management gradient. Fungal communities were considered likely contributors to variation in mVOC emissions, as fungal genera showed stronger correlations with indicative mVOC compounds than bacterial genera did (Chapter 4), though the direction and nature of this relationship, whether reflecting production, consumption or co-response to shared drivers, could not be established from this data alone. The co-variation between fungal community composition, SOC and decomposition dynamics along the management gradient suggests that these variables share a common axis of variation, consistent with the recognised role of fungi in carbon cycling in permanent grasslands (Li et al., 2019). This could also have a strong influence on the metabolic activity in the soil and, consequently, the mVOC profile (Insam & Seewald, 2010) (Chapter 4). However, the results from my study shows evidence for these associations, and other experimental approaches would be needed to establish the direction and magnitude of these relationships in agricultural soils.

Fungi have shown to be more sensitive to nutrient inputs and soil disturbance in grasslands, owing to their different physiological nutrient requirements, sensitivity to physical disturbance and more intricate associations with plants (Maharning et al., 2009). It was also suggested by Seaton et al. (2022) that changes in the fungal community may prove a stronger indicator for soil conditions in grasslands than changes in bacterial community

composition. One limitation of Chapter 3 and 4 is that we did not measure biomass of bacteria and fungi. Greater bacterial or fungal biomass could indicate a stronger influence of either community on the measured soil processes and mVOC profiles in the soils (Leff & Fierer, 2008; Singh & Gupta, 2018) , although this is not always the case (Ma et al., 2022). Notably, bacterial community composition showed a significant association with respiration despite showing no clear response to management intensity (Chapter 3; Thomson et al., 2010), suggesting that bacteria may influence soil functioning through processes that are not directly structured by management.

Despite the clear responsiveness of fungal community composition to variations in management intensity, the extent to which functional capabilities of these communities differed along the management gradient remained less evident. Fungal genera that were indicative of changes in management intensity along the gradient, tended to decrease in abundance with increasing extensification (Chapter 4). Although not shown in the chapters, analyses of functional guild composition did not reveal clear shifts in functional groups of the fungal community composition analysed in Chapters 3 and 4 (see Suppl. Information of those chapters). This apparent discrepancy may reflect limitations in current functional annotation databases of fungi, which remain incomplete for many fungal taxa. Improving methods to infer functional capacity from microbial community composition therefore remains an important research priority (Krause et al., 2022; Nguyen et al., 2016)

Soil pH as an abiotic condition

In parallel with fungal-associated axis, soil pH emerged as a strong correlative variable across multiple chapters, showing significant associations with microbial community composition, enzymatic activity and mVOC profiles (Chapters 3 and 4). These associations suggest that in our studies, pH may influence which organisms and processes can operate under given soil conditions. The importance of pH in structuring soil biological and chemical processes has been well established (Husson, 2013). Numerous studies have identified soil pH as a major predictor of microbial diversity, community composition and activity (George et al., 2019; Griffiths et al., 2011; Hendershot et al., 2017).

Empirical evidence across chapters indeed shows the predictive capacity of soil pH for soil communities and processes. In chapter 3, soil pH was identified as a key predictor of extracellular enzymatic activity, independent of management intensity, and showed significant associations with microbial community composition. Although pH often has been reported to be more strongly associated with bacterial than fungal communities (George et al., 2019; Seaton et al., 2022), the results of this thesis indicate significant relationships with fungal community composition as well. Similarly, in chapter 4, soil pH showed the

strongest correlative associations with multiple mVOC classes. A possible explanation is that pH directly influences the metabolic activity and community composition of the fungi and bacteria that produce these volatiles, as microbial volatile production is known to be sensitive to soil chemistry (Schulz-Bohm et al., 2017). However, disentangling the direct effect of pH on VOC production from its indirect effects via bacterial and fungal community composition requires experimental approaches beyond what was possible in this field study.

These findings suggest that soil pH functions primarily as a physicochemical boundary condition; one that determines which organisms and processes can operate under given soil conditions rather than as a variable that tracks or drives management-driven reorganisation. In this sense, pH does not need to co-vary with management intensity to play an important role: it sets the context within which biological communities respond to management, and different pH conditions may therefore lead to different magnitudes or directions of biological response to the same management gradient. This interpretation is consistent with the observation that pH displayed a bimodal distribution across fields that did not correspond to predefined management categories. For example, both conventional and extensive farmers may either lime or refrain from liming, depending on their individual goals and soil conditions (Gibbons et al., 2014). The role of pH as a variable deserves explicit consideration when studying soil responses to management gradients. Soil pH in agricultural systems is shaped by opposing forces: intensive fertilisation with ammonium-based or mineral nitrogen fertilisers tends to acidify soils over time through nitrification and base cation leaching (Goulding, 2016), while organic manure inputs can buffer against this acidification (Kidd et al., 2017). At the same time, farmers routinely counteract acidification through liming, independently of their overall management intensity (Goulding, 2016). This creates an interpretive ambiguity: the observed pH of a field reflects not only the acidifying or buffering effect of its fertilisation regime, but also the individual liming decisions of the farmer. Why individual farmers choose to lime or refrain from liming was not captured in this study, but the resulting pH variation, independent of management category is consistent with liming being an individual agronomic decision rather than a management-type specific practice (Gibbons et al., 2014) As a result, pH cannot serve as a straightforward indicator of extensification state in the way that fungal community composition or SOC potentially can. Rather, it is best understood as a contextual variable that modulates the biological response to management, but whose value at any given moment reflects a combination of soil type, fertilisation legacy and active agronomic intervention (Goulding, 2016) that cannot be fully disentangled in observational field studies.

Interaction between biological and abiotic factors

The co-variation of fungal community composition and soil pH with patterns of soil reorganisation along the management gradient offers a conceptual framework for understanding why soils do not respond uniformly to management intensity. Rather than representing equal and interchangeable dimensions, these two axes appear to operate at different levels: soil pH varies as a physicochemical boundary condition that shapes which organisms and processes can operate under given soil conditions, while fungal community composition co-varies with carbon cycling variables (SOC and decomposition) along the same management gradient. Together, these two axes of co-variation account for a substantial proportion of the observed variation in soil biological communities and functional processes across the gradient, and recognising their distinct roles helps explain why different soil components show divergent responses to the same management intensity.

Measuring soil extensification: multifunctionality and mVOCs

The results of this thesis research show that precise measurement of soil multifunctioning is a non-trivial challenge, because different indicators of soil functioning respond to different changes that take place in soils during transition from intensively to extensively used. The various aspects of the soil properties do not necessarily change into in the same direction.

The results of chapter 3 revealed that not all soil processes respond to management intensity in the same direction or with the same sensitivity. This divergence challenges a common assumption in soil ecology: that changes in management propagate through biological communities to influence multiple ecosystem processes coherently (Delgado-Baquerizo et al., 2020). In particular, litter decomposition showed clearer associations with management intensity and microbial composition than laboratory-based proxies such as extracellular enzymatic activity or substrate-induced respiration. These differences reflect the complex nature of soil functioning, which can be measured at multiple organisational levels, from community composition through metabolic activity to realised ecosystem processes. These do not necessarily respond in parallel. Abiotic constraints such as pH, or differences in measurement context between field and laboratory conditions, can decouple microbial composition from functional expression, meaning that a shift in community composition does not automatically translate into a corresponding shift in measured process rates.

These divergent responses have direct implications for how soil multifunctionality is assessed. Multifunctionality indices, as commonly applied, aggregate normalised values of

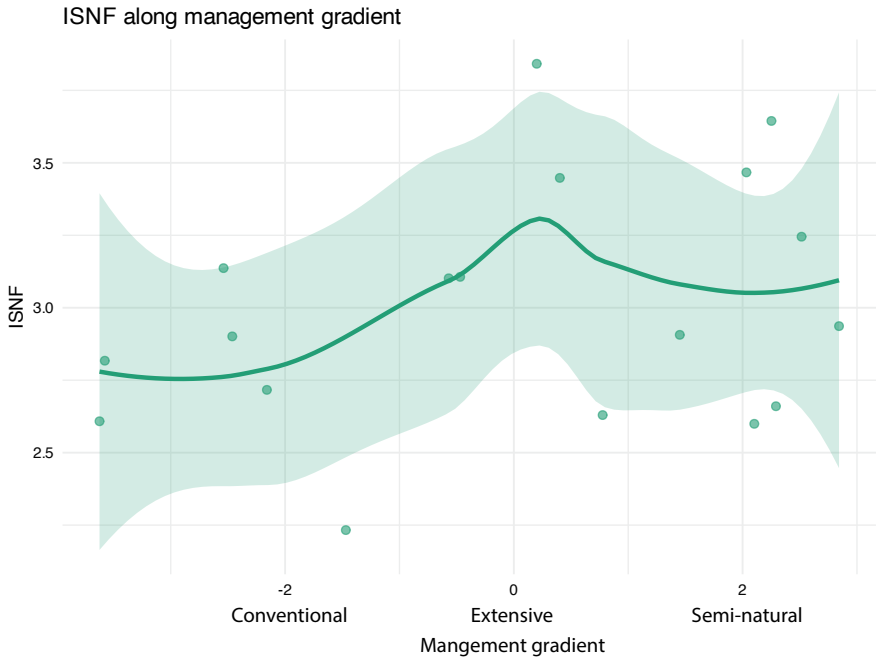


Figure 8.3: Relationships between the integrated soil multifunctionality index (ISNF) and the management gradient were evaluated using locally weighted polynomial regression (LOESS). Mean ISNF values per field were plotted against the management gradient (PC1 values), and a smoothed trend with 95% confidence intervals was fitted to visualize potential non-linear patterns. Individual observations were displayed as individual data points.

multiple processes directly into a single composite score, implicitly treating all processes as equivalent contributions (Byrnes et al., 2014; Delgado-Baquerizo et al., 2020; Manning et al., 2018). This aggregation skips a conceptually important intermediate step: before combining processes from different functions into a multifunctionality score, it would be informative to first inspect how processes that underpin the same function behave relative to one another; a multi-process, single-function approach. This matters because processes within a single function can diverge, and that divergence disappears when they are averaged together with processes from other functions. Byrnes et al. (2014) already warned that averaged multifunctionality indices cannot distinguish between a scenario where all processes perform at intermediate levels and one where some perform at high levels while others perform poorly. The results of Chapter 3 illustrate this explicitly: decomposition, enzymatic activity and respiration all underpin nutrient cycling, yet they responded differently to management intensity. Aggregating these directly into a multifunctionality score would obscure this within-function divergence entirely.

To visualise this, Figure 8.3 shows an integrated soil nutrient functioning index (ISNF),

which combines the normalised values of these three processes (all underpinning the same function of nutrient cycling) across fields along the management gradient (see Suppl. Methods). This index is neither proposed as a novel multifunctionality metric, nor does it claim to be more informative than existing approaches. Rather, it illustrates what a function-level aggregation step looks like in practice, and how the resulting pattern compares with the individual process responses shown in chapter 3. The ISNF shows a gradual positive trend with decreasing management intensity, consistent with the gradient-based interpretation developed throughout this thesis. However, as the individual process results in chapter 3 show, this overall trend masks meaningful divergence among the underlying processes, precisely the kind of information that is lost when aggregation proceeds directly from processes to multifunctionality without an intermediate function-level inspection.

mVOCs as proxies for monitoring extensification transitions

A central challenge in monitoring agricultural extensification is identifying indicators that can capture meaningful changes in soil communities and functioning across management contexts that are sensitive enough to detect changes. Metabolic indicators such as microbial volatile organic compounds (mVOCs) could be such potential indicators (Abis et al., 2020; Brown et al., 2021). These volatiles are low-molecular-weight compounds produced by soil microbes during primary and secondary metabolism. These volatiles have been proposed as integrative proxies to monitor shifts in soil microbial communities, because their profiles reflect the combined diversity, abundance and activity of the microbial community (Brown et al., 2021; Insam & Seewald, 2010). Across the chapters of this thesis, the potential and limitations of mVOCs as monitoring proxies were examined from three complementary angles: their sensitivity to management variation along the extensification gradient (Chapter 4), their comparative performance against established biochemical proxies in supervised classification (Chapter 6), and, from a more exploratory users feasibility survey perspective, the practical requirements identified by farmers and soil scientists for a useful soil-life sensor (Chapter 5). Together, these perspectives reveal both the potential and the specificity of conditions under which mVOCs can serve as meaningful monitoring tools for detecting shifts in soil microbial community composition and activity.

The overall mVOC profile showed high within-type variability and pairwise differences between predefined land use categories, which were not statistically significant after correction for multiple comparisons. Still, a Threshold Indicator Taxa Analysis (TITAN) identified sixteen compounds (from a total of 75) whose abundances changed systemat-

ically along the continuous management gradient (Chapter 4). Chapter 6 extended this analysis by directly comparing mVOC profiles against extracellular enzymatic activities. This is a well-established proxy for soil microbial functioning that was tested for its use to classify management categories. In unsupervised ordination, neither mVOCs nor enzyme activities showed clear separation among land use categories, consistent with the high within-type variability documented in chapter 4 and with the gradient-based view that soil functioning does not map neatly onto discrete categorical boundaries. However, when targeted supervised classification was applied using machine-learning classifiers trained on the post-priori management labels, mVOC profiles achieved a higher balanced accuracy than enzyme activities across three land use classes (Chapter 6, Fig. 6.5).

First, these findings have direct implications for the potential of mVOCs as proxies. The failure of mVOC profiles to separate management categories in unsupervised ordination did not indicate an absence of ecological information. Rather, it indicated that this information is distributed across many variables and their interactions instead of being concentrated along a few principal axes (Chapter 4). This phenomenon hints at the potential of mVOCs to be used as a proxy for tracking transitions in land use, because the relevant signal is useful for subtle changes, instead of for indicating discrete jumps between states. That the discriminatory power only became apparent under supervised, non-linear machine-learning classification (Chapter 6) which further supports this interpretation: management boundaries in mVOC space are complex and curved, and do not follow the kind of linear separation that ordination approaches are designed to detect.

Second, the findings from chapter 4 and 6 reinforce the point that mVOC profiles cannot be interpreted in isolation from the physicochemical environment in which they are produced (Leff & Fierer, 2008; Schulz-Bohm et al., 2017) and that monitoring applications will require explicit consideration of abiotic context in combination with metabolic signal. Interestingly, as already mentioned, the mVOCs were related to the same key variables from chapter 3; fungal communities and pH. In addition, the most discriminating mVOC pairs identified in the classification analysis (dimethyl sulphide combined with methyl acetate or methyl benzoate, and heptanal combined with dimethyl sulphide) point at coordinated shifts in sulphur cycling and fungal-associated metabolic activity as key signals of variations in soil management. If the potential of mVOCs as monitoring tools would be further investigated, it would be recommendable to also collect a range of abiotic and biotic factors alongside mVOC samples. To my knowledge, the study in chapter 4 and 5 was the first of its kind in agricultural grasslands. These results, based on a single study area, may need to be verified in other areas with different soils, which could give different outcomes. For example, sandy soils might have different effects on mVOCs than the clay soils that are present

in Ooijpolder.

Exploratory interviews with farmers and scientific stakeholders (Chapter 5) revealed monitoring needs that closely parallel the gradient framework developed in this thesis. Farmers, particularly those in conventional systems actively adjusting their practice, wanted to know whether their soils were responding to gradual management changes. This preference for trajectory-sensitive feedback aligns with the observation from chapters 4 and 5 that mVOC dynamics are most sensitive at intermediate points along the extensification gradient. From a scientific perspective, scientific stakeholders at the workshop during second symposium of the National Centre of Soil Ecology (NCSE) added that sensors should connect VOC patterns to process-relevant ecological signals, such as mineralisation, decomposition, disease suppression, rather than deliver chemical fingerprints alone. This suggestion is consistent with the conclusions in my thesis that predictive power will be greatest when indicators can be linked to specific biological mechanisms and processes. Realising this potential will require dedicated research efforts, including the development of mVOC reference databases under defined soil and management conditions, and studies that explicitly link microbial community composition, functional processes and mVOC profiles across both semi-controlled and field settings.

Taken together, the evidence from Chapters 4, 5 and 6 supports an initial assessment of mVOCs as monitoring proxies in field conditions. Their integrative character reflecting community composition, abundance and metabolic state simultaneously constitutes a functional advantage over single-process indicators, particularly for capturing the kind of multidimensional soil reorganisation described in the preceding sections of this synthesis. Their sensitivity to variation along the management gradient makes them more appropriate for detecting differences in current management state than for confirming categorical management, a property that aligns well with the gradient-based framework developed throughout this thesis and with the monitoring needs expressed by practitioners (Chapter 5). It should be noted, however, that this sensitivity was demonstrated in a cross-sectional study design, and whether mVOC profiles are equally sensitive to temporal change within individual fields during an ongoing transition remains to be tested. At the same time, the context dependence of mVOC signals implies that they need to be interpreted within a physicochemical and biological framework of the study area.

The findings throughout this thesis suggest a specific and complementary possible role for mVOCs within a multi-indicator monitoring framework. First, as early-response metabolic indicators that could detect functional changes before community composition shifts become statistically discernible. Second, as integrative fingerprints that capture

emergent metabolic interactions not accessible through enzyme assays or community sequencing alone. Realising this potential will require standardisation of field sampling and analytical protocols, expansion of reference databases, such as mVOC library 4.0 (Lemfack et al., 2014), and integration with the physicochemical and biological context variables that drive mVOC production and consumption in agricultural soils. Progress on these aspects would substantially advance the case for mVOCs as a component of the trajectory-sensitive, multi-proxy monitoring framework that the results of this thesis call for.

From gradients to action perspectives: limits revealed by inoculation

Observational gradients provide insight into how soils are reorganised across management contexts, but they do not automatically imply that soils can be steered along the same axes through active intervention. The inoculation experiment described in Chapter 7 evaluates whether observed gradient patterns can be translated into actionable management interventions. If the mechanisms underlying gradient-associated transitions in soil properties and functioning were readily transferable, introducing microbial inocula could shift soil communities measurably along similar trajectories. However, after two years of treatment, no significant differences in microbial community composition were detected between inoculated and non-inoculated field plots, while temporal variation and plant community treatments exerted stronger influences (Chapter 7).

Several factors may explain this apparent discrepancy between the variations of soil properties along the observational gradients (Chapter 3) and the absence of treatment effect in the inoculation study (Chapter 7). First, differences in soil type between the gradient studies (loamy clay soils) and the inoculation experiment (loess soils) complicate direct comparisons, as soil texture influences microbial community establishment and inoculum survival (Bach et al., 2010). Second, timescale likely plays a critical role, as the grasslands studied along the management gradient had experienced at least a decade of consistent management, whereas the inoculation experiment spanned only two years. Based on the results of Chapters 3 and 4, a detectable shift in fungal community composition would have been the most sensitive indicator of a system in transition. However, two years may have been insufficient for fungal hyphal networks to establish and for plant-soil feedbacks of the mixed plant communities to develop to a degree that restructures the broader microbial community (Luo et al., 2023; Zhang et al., 2021). This interpretation is consistent with findings from other studies, which also report slow community responses following extensification (Morriën et al., 2017; Wubs et al., 2019a). Notably, experiments in which the resident soil community was removed or disrupted prior to inoculation, through topsoil re-

moval (Wubs et al., 2016) or sterilisation (De Deyn et al., 2004), indeed showed detectable community shifts within shorter timeframes. This suggests that the resident microbial community suppresses colonisation by introduced soil biota when left intact, slowing the recovery trajectory. Finally, soil pH showed relatively limited variation among treatments in the inoculation experiment and fell within the lower range observed in Chapter 3, which may have constrained the compositional shifts in microbial communities that inoculation was intended to promote.

Importantly, although microbial community composition did not shift detectably, functional changes may still have occurred at metabolic levels not captured by the measurements in this experiment. Individual microbial taxa can possess multiple functional capabilities and shift their metabolic expression depending on environmental conditions, a phenomenon known as functional plasticity (Li et al., 2026), meaning that compositional stability does not preclude functional change. Metabolic indicators such as mVOCs could potentially reveal early functional responses preceding compositional changes, highlighting a direction for future mVOC research (Chapter 4 and 5).

Overall, these findings emphasise an important distinction between understanding ecological patterns and being able to manipulate them: observed gradients provide insight into system organisation, but they do not automatically imply that soil systems can be steered along the same axes within short management timeframes.

From understanding to implementation: transdisciplinary perspectives on agricultural transitions

Although ecological mechanisms are central to understanding soil responses to management, transitions toward sustainable agricultural systems cannot be achieved through ecological knowledge alone (IPBES, 2025). Agricultural management decisions are also shaped by economic constraints, policy frameworks, social norms and historical land-use trajectories, all of which influence whether and how farmers may adopt new practices. Consequently, improving soil functioning through extensification requires not only mechanistic insight into soil processes, but also integration with socio-economic factors. For instance, the results of this study show what can change when a farmer decides to transition towards more extensive practices (Chapter 3), yet it does not look into what a farmer needs to make that transition (e.g. finances, social support (Kleijn et al., 2019; Witvliet et al., 2024). In addition, other social-historical contexts could be important for the entire region when it comes to changing managing practices (Baumgarten et al., 2025)

One important challenge lies in translating scientific knowledge into forms that are

meaningful and actionable for practitioners. The complexity of soil systems identified in this thesis (including multidimensional responses, factor-dependent predictability and context-specific constraints) contrasts with the need for simple and practical guidance in agricultural decision-making (Bouma, 2014). Bridging this gap requires approaches that integrate ecological indicators with stakeholder priorities and management objectives, moving beyond purely disciplinary perspectives. Transdisciplinary research frameworks offer opportunities to address this challenge by involving stakeholders in the co-development of monitoring tools and management strategies (Roux et al., 2017). Such approaches recognise that farmers possess experiential knowledge about their land and that successful transitions depend on aligning scientific recommendations with practical feasibility and economic viability. Chapter 5 represents a first step in this direction, by involving farmers and scientific stakeholders in identifying the practical requirements for a useful soil monitoring tool. Monitoring tools that function as "boundary objects," connecting scientific understanding with management decisions, may help facilitate communication and adoption of sustainable practices (Wood & Blankinship, 2022).

Policy contexts also play a critical role in shaping transitions. Certification schemes and regulatory frameworks often rely on categorical definitions of management systems, which may not align with the gradient-based ecological reality demonstrated in this thesis. Developing policies that accommodate gradual transitions and recognise intermediate stages of extensification could support more flexible and realistic pathways toward sustainability. At the same time, economic incentives and institutional support remain essential to enable farmers to implement management changes that may initially reduce productivity or increase uncertainty. Integrating ecological, social and economic perspectives is therefore essential for designing agricultural transitions that are both environmentally effective and socially feasible (Baumgarten et al., 2025; Witvliet et al., 2024). The mechanistic insights generated in this thesis provide a foundation for such integration by clarifying how management influences soil systems and identifying the conditions under which interventions may succeed or fail. For example, the co-variation of fungal community composition with SOC and decomposition along the management gradient suggests that management practices promoting fungal community development may support the transition toward more extensive soil functioning (Hartmann et al., 2015). However, a farmer needs to be facilitated in that transition with knowledge on how to do so effectively. Future research should continue to build on this foundation through interdisciplinary collaborations that combine ecological experimentation, socio-economic analysis and stakeholder engagement(Leander Oh et al., 2026).

Understanding soil reorganisation determines prediction and manageability

Agricultural extensification reorganises soils through interacting biological and abiotic factors that differ in sensitivity, temporal dynamics and constraints. The central insight of this thesis is that, in our research, fungal community dynamics and soil pH jointly structure soil reorganisation along the management gradient that was used for the present thesis research, shaping which soil properties respond to extensification and which indicators are most informative for monitoring that response. Conceptualising extensification as a development along a multidimensional management gradient, rather than a shift between discrete categories, provided a more realistic representation of both farming practice and soil ecological responses. Within this framework, soil functioning cannot be fully captured by single indicators or aggregated multifunctionality metrics without considering the underlying mechanisms. Predictability depends on the dominance of specific factors and the extent to which abiotic constraints modulate biological processes. The inoculation experiment suggested that actively steering soil communities through inoculation will be a slow process, and that when trying to steer, meaningful shifts in community composition may require timescales well beyond what short-term interventions can achieve. Monitoring approaches that focus on trajectories of change, integrate multiple indicators and account for environmental context will be more appropriate for assessing soil transitions than static threshold-based assessments. Implementing such approaches requires collaboration across disciplinary boundaries and genuine engagement with stakeholders, so that the mechanistic insights generated here can be translated into practical applications. This thesis provides a foundation for that work, built on the recognition that improving soil functioning in agricultural systems depends on understanding how management reshapes the interactions among biological communities, soil properties and ecosystem processes, and on being transparent about where the limits of that understanding lie.



Photograph taken at the author's family farm in Zeeland, the Netherlands.

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Photograph taken at the author's family farm in Zeeland, the Netherlands.

Summary

The success story of modern agriculture - its capacity to feed billions of people - has recently become the source of its greatest challenge: producing food sustainably without harming the environment. Post-war intensification (mineral fertilisers, synthetic pesticides, high-yielding varieties) dramatically raised productivity, but at the cost of nutrient surpluses, biodiversity loss, and the erosion of the ecological base farming itself depends on. The Netherlands exemplifies this pattern: more than half its land surface is used for agriculture, mostly feeding a dairy sector that generates economic value but also produces some of Europe's highest nitrogen surpluses. Recently, the societal cost of the dutch agricultural sector was estimated at roughly €18.6 billion per year, while its economical worth was estimated at €13,3 billion per year. The central challenge is therefore no longer whether agriculture can produce enough food, but whether it can do so without undermining the ecological systems it depends on.

Extensification - reduced fertiliser and manure inputs, lower livestock densities, reduced nutrient losses - is a promising alternative, shifting productivity from external inputs toward internally regulated biological processes such as nutrient cycling, pest control, and soil structure formation. Permanent dairy grasslands are especially promising candidates for this shift, since they retain continuous plant cover and limited soil disturbance while also delivering ecosystem services such as climate and water regulation. However, agricultural soils carry the legacy of decades of intensification in their physical, chemical and biological properties, which may limit how much ecological benefit a grassland can deliver once management changes - understanding these constraints requires turning attention belowground.

In this thesis, I organised my research around three questions: (1) how does grassland management intensity affect soil microbial communities, soil properties and soil functioning; (2) can such changes be detected using a novel proxy based on microbial volatile organic compounds (mVOCs); and (3) can soil inoculation accelerate the transition toward more extensive grassland. Questions 1 and 2 were studied using an 18-field management

gradient in the Ooijpolder, a riverine landscape near Nijmegen, the Netherlands, where fields share soil type, topography and climate but differ in management history, within the transdisciplinary Living Lab Ooijpolder-Groesbeek project. Question 3 was addressed in a separate field experiment near the village of Groesbeek, the Netherlands.

Representing management as a category or a gradient

A recurring discussion in ecology is whether management is best represented as a continuous gradient or as discrete categories such as conventional, extensive and semi-natural. In chapter 2, I compared a priori management categories, assigned by farmers themselves, against data-driven post-priori clusters based on management variables derived from questionnaires (mowing, grazing, fertilisation, grassland and management age) and measured field variables (plant species richness). The match between a-priori and post-priori was imperfect: several fields labelled "extensive" clustered closer to "semi-natural" once actual management data were used, showing that a category based on a farmer's own perception of management can obscure variation underneath it.

I then tested whether management intensity itself was better captured by a continuum (gradient) or discrete clusters, validating both against an independent, remote-sensing based proxy of land-use intensity. All three approaches explained a similar amount of variation, but statistical tests found no clear group separation, leading to the conclusion that variation in long-term management was best presented as a gradient.

Building on this gradient framing, chapters 3 and 4 examined the consequences of long-term management variation for soil functioning. I measured biotic properties (bacterial and fungal community composition), abiotic properties (carbon and nitrogen content, pH, bulk density), and three processes underpinning nutrient cycling: litter decomposition (using standardised tea bags), extracellular enzymatic activity (how actively the microbial community breaks down substrates), and substrate-induced respiration, measured as the evenness of substrate use (MicroResp). Not all of these responded to management in the same way: fungal community composition, soil organic carbon, and decomposition rate were all strongly associated with the gradient, while bacterial composition, bulk density, nitrogen, enzymatic activity, and evenness of substrate use showed weak or inconsistent relationships with it. Long-term management, in other words, does not push the soil system in one direction but specific variables respond in different ways.

Fungal and bacterial communities were correlated with each other but responded quite differently to the gradient: fungal composition was the more responsive of the two and more strongly linked to shifts in soil functioning overall, while bacterial composition was not significantly affected by the gradient itself, though it still correlated with the evenness

of substrate use. Soil organic carbon showed the strongest response of all abiotic variables, increasing under extensive management and correlating negatively with manure inputs and livestock density - consistent with more plant residue returning to soil under lower-intensity management, and closely tied to fungal composition and decomposition rate. Together, these three variables; SOC, decomposition rate, and fungal community composition, emerged as robust indicators of current management state.

Soil pH told a different story: rather than tracking management intensity, it was more likely shaped by individual farmers' liming decisions, irrespective of management. pH was the strongest predictor of enzymatic activity, which itself was not linearly related to the gradient. In our study, this implied that enzymatic activity could not be used as a simple indicator of long-term management intensity.

Measuring soil function: why aggregated multifunctionality indices can be misleading

The variation in response of the three processes underpinning one function in chapter 3, matters for how we monitor soil functioning. In many studies, multifunctionality indices are calculated by relating one or two processes to a function and averaging those functions into an index. I argue that we need to be cautious which processes we select, even within a function. It is worth first establishing whether the processes underpinning a single function respond in similar ways.

mVOCs as a potential monitoring proxy

Microbial volatile organic compounds (mVOCs) (metabolites produced by soil bacteria and fungi) have recently been proposed as a proxy for monitoring the metabolic functioning of soil microbial communities. Chapters 4, 5 and 6 tested this from three angles: sensitivity to the management gradient, practical usability, and performance against an established proxy, enzymatic activity.

In chapter 4, the complete mVOC profile did not separate cleanly between land-use types, largely due to high within-type variation. Using a targeted statistical analysis (TITAN2), I identified sixteen compounds that specifically tracked the gradient. As in chapter 3, fungal (but not bacterial) community composition and soil pH again emerged as the strongest associated soil factors, reinforcing their role as a shared driver across chapters. A more cautionary finding: known relationships between specific mVOC compounds and their microbial producers, drawn from laboratory-based reference databases, mostly did not hold up in this field setting, underlining how context-dependent these signals become

once studied in situ.

In chapter 6, I compared mVOC profiles directly against enzymatic activity (an established proxy). Neither proxy separated cleanly between land-use types, and the patterns they picked up did not closely resemble each other. I interpreted this not as a failure of either proxy, but as a sign that soil functioning does not map neatly onto land-use categories in the first place. Despite this, mVOCs and enzyme activities shared a strong underlying gradient, and, contrary to what I expected, mVOCs outperformed enzyme activity when classifying management category. This is consistent with chapter 3's finding that enzymatic activity, unlike fungal composition, showed little association with the gradient to begin with.

Chapter 5 combined farmer interviews with a workshop of soil researchers to ask what a usable soil-life sensor would need to do. Interest was highest among conventional farmers, who wanted a tool that could tell them whether their ongoing management adjustments were having an effect, therefore prioritising actionable, comparative information (spatial variability, early warnings, benchmarking) over simply confirming a category label of conventional versus extensive or organic. Researchers converged on a similar point: a useful sensor needs to link volatile signatures to actual ecological processes, not just deliver a chemical fingerprint, while flagging real obstacles such as limited reference databases.

Together, these chapters suggest mVOCs have genuine promise as an early, integrative proxy for tracking whether (and in which direction) a soil is changing. Realising this will require validation across other soil types, reference benchmarks, and repeated sampling over time, rather than the single time-point design used here.

Understanding a gradient doesn't mean you can steer it

The final question - whether the transition can be accelerated - was addressed in chapter 7, combining a greenhouse experiment with a two-year field trial on whole-soil inoculation using donor soil from herb-rich reference sites. In the greenhouse, inoculation with fresh donor soil clearly and consistently steered both prokaryotic and fungal communities toward the donor community, with inoculation explaining more variation than seed mixture, indicating the living biota in the inoculum, not abiotic differences, drove the shift.

In the field, however, no comparable shift was detected after two years, regardless of seed mixture; communities in both inoculated and control plots drifted gradually toward the donor community regardless of treatment, likely reflecting the broader succession of arable-to-grassland conversion rather than an inoculation effect. Neither experiment showed a significant shift in microbial functional groups. Several factors likely explain

this contrast: a thin inoculum layer chosen for practicality, limited prior soil disturbance, and a timeframe possibly too short relative to the six-plus years other studies suggest is needed. Overall, whole-soil inoculation shows genuine potential to steer microbial communities under controlled conditions, but this did not translate into a fast, reliable effect under realistic field constraints - suggesting the approach still has potential for practice, but likely needs a longer time horizon and complementary measures such as greater inoculum quantity or prior soil disturbance.

The transdisciplinary context

I close by locating these ecological findings within a socio-economic and policy context. Knowing what changes belowground under extensification is not the same as knowing what a farmer needs, financially or socially, to make that transition. Certification schemes and most policy frameworks still rely on categorical definitions of management, which sit uneasily with this thesis's central finding that a gradient captures more of the variation in soil responses. The farmer and stakeholder interviews in chapter 5 are a first step toward monitoring tools that connect scientific insight with the practical, gradual way farmers actually adjust their management.

Overall takeaway

The throughline of this thesis is a shift in framing: extensification is not a switch between categories but a trajectory, enacted through soil properties and communities that respond at different speeds and are shaped by different mechanisms. This has three concrete outcomes: it identifies SOC, decomposition rate and fungal community composition as robust indicators of current management state; it positions mVOCs as a promising but context-dependent monitoring proxy suited to tracking gradual change rather than confirming a fixed category; and, through the inoculation experiment, it shows that understanding a gradient's structure is not the same as being able to steer a field along it quickly. Together, these findings argue for monitoring built around trajectories and multiple, context-aware indicators, and for policy that can accommodate gradual, continuous transitions rather than only categorical ones.



Photograph taken at the author's family farm in Zeeland, the Netherlands.

Nederlandse samenvatting

Het succesverhaal van de moderne landbouw – het vermogen om miljarden mensen te voeden – is tegelijkertijd de bron geworden van haar grootste uitdaging: voedsel duurzaam produceren zonder schadelijke impact op het milieu. Naoorlogse intensivering (minerale meststoffen, synthetische bestrijdingsmiddelen, hoogproductieve rassen) verhoogde de productiviteit drastisch, maar ging gepaard met nutriëntenoverschotten, verlies aan biodiversiteit, en de aantasting van de ecologische basis waarop de landbouw zelf steunt. Nederland is een voorbeeld van dit patroon: meer dan de helft van het landoppervlak wordt gebruikt voor landbouw, waarvan het grootste deel dient ter voeding van een zuivelsector die economische waarde genereert, maar ook een van de hoogste stikstofoverschotten van Europa produceert. De maatschappelijke kosten hiervan – voor bodem, water, natuur, klimaat en gezondheid – zijn recent geschat op ongeveer €18,6 miljard per jaar, tegenover geschatte baten van €13,3 miljard per jaar. De centrale uitdaging is dan ook niet langer of de landbouw voldoende voedsel kan produceren, maar of dit kan zonder de ecologische systemen te ondermijnen waarop zij afhankelijk is.

Extensivering – de vermindering van externe inputs zoals kunstmest, lagere veedichtheden, verminderde nutriëntenverliezen – is een veelbelovend alternatief, waarbij de productiviteit verschuift van het gebruik van externe inputs naar intern gereguleerde biologische processen zoals het afbreken van organische stof en het recyclen van nutriënten. Permanente melkveegraslanden zijn geschikte systemen voor extensivering, omdat ze een permanent gewas hebben waarbij de bodem minder wordt verstoord, terwijl ze ook ecosysteemdiensten leveren zoals klimaat- en waterregulatie. Landbouwbodems dragen de sporen van decennia aan intensief gebruik met zich mee, in hun fysische, chemische en biologische eigenschappen. Die geschiedenis kan ervoor zorgen dat een grasland minder snel ecologisch herstelt, ook als het beheer verandert. Daarom is het belangrijk om te kijken naar wat er zich in de bodem zelf afspeelt.

In dit proefschrift heb ik mijn onderzoek georganiseerd rond drie vragen: (1) hoe beïnvloedt de intensiteit van graslandbeheer bodemmicrobiële gemeenschappen,

bodemeigenschappen en bodemfunctioneren; (2) kunnen dergelijke veranderingen worden gedetecteerd met behulp van een nieuwe proxy gebaseerd op microbiële vluchtige organische stoffen (mVOCs); en (3) kan bodeminoculatie de overgang naar een extensiever grasland versnellen. Vraag 1 en 2 zijn onderzocht aan de hand van een managementgradiënt op basis van het beheer van 18 graslanden in de Ooijpolder, een rivierenlandschap nabij Nijmegen, Nederland, waar percelen hetzelfde bodemtype, dezelfde topografie en hetzelfde klimaat delen, maar verschillen in beheersgeschiedenis. Dit alles speelde zich af binnen het transdisciplinaire project Living Lab Ooijpolder-Groesbeek. Vraag 3 is onderzocht in een afzonderlijk veldexperiment nabij het dorp Groesbeek, Nederland.

Beheer weergeven als categorie of als gradiënt

Een terugkerende discussie in de ecologie is of je beheer het beste kunt weergeven als een doorlopende gradiënt (bijvoorbeeld van minste naar meest intensief) of als losse categorieën, zoals conventioneel, biologisch, extensief en half-natuurlijk. In hoofdstuk 2 heb ik dit onderzocht. Eerst keek ik naar hoe boeren hun eigen percelen indeelden op basis van categoriën (”gangbaar”, ”extensief”, ”half-natuurlijk”). Vervolgens maakte ik, los daarvan, een indeling op basis van de daadwerkelijke beheersgegevens: hoe vaak er gemaaid en beweid werd, hoeveel er bemest werd, hoe oud het grasland was, en hoe rijk aan plantensoorten het perceel was. Deze twee indelingen kwamen niet helemaal overeen: verschillende percelen die door de boer als ”extensief” waren bestempeld, bleken op basis van de daadwerkelijke gegevens juist dichter bij ”half-natuurlijk” te zitten. Dit laat zien dat hoe een boer zijn eigen beheer noemt, niet altijd overeenkomt met hoe intensief dat beheer werkelijk is.

Daarna heb ik getoetst of de intensiteit van beheer beter kan worden gerepresenteerd door een gradiënt of door groepen. Om dit te controleren, vergeleek ik beide met een onafhankelijke maat voor landgebruiksintensiteit op basis van satellietbeelden. Alle drie de manieren van indelen (de indeling van de boeren zelf, de datagedreven groepen, en de gradiënt) verklaarden ongeveer evenveel van de variatie. Maar statistisch gezien bleek er geen duidelijke verschil te zijn tussen groepen: percelen liepen geleidelijk in elkaar over. Daarom concludeer ik dat beheer, in mijn studie, het best kan worden weergegeven als een gradiënt, niet als losse hokjes.

Voortbouwend op deze gradiënt onderzochten hoofdstuk 3 en 4 het effect van langdurige verschillen in beheer op hoe de bodem functioneert. Ik heb gekeken naar levende eigenschappen van de bodem (welke bacteriën en schimmels erin voorkomen), naar chemische eigenschappen (hoeveel koolstof en stikstof erin zit, de zuurgraad, en hoe

verdicht de bodem is), en naar drie processen die allemaal met de kringloop van voedingsstoffen in de bodem te maken hebben: hoe snel plantenmateriaal wordt afgebroken (gemeten met theezakjes die in de grond worden begraven), hoe actief de bodem is in het afbreken van stoffen via enzymen, en hoe breed het scala aan koolstofbronnen is dat de bodemgemeenschap kan gebruiken (gemeten met de MicroResp-methode). Niet al deze eigenschappen reageerden op dezelfde manier op beheer: schimmelgemeenschap(welke schimmels er voorkomen), de hoeveelheid organische koolstof, en de afbraaksnelheid van plantenmateriaal hingen allemaal duidelijk samen met de gradiënt, terwijl bacteriegemeenschap(welke bacterieën er voorkomen), bodemdichtheid, stikstof, enzymactiviteit en de breedte van koolstofgebruik zwak of wisselend met elkaar samenhingen. Langdurig beheer zorgt in de bodem dus niet voor één duidelijke richting: verschillende onderdelen van het bodemsysteem reageren elk op hun eigen manier.

Schimmel- en bacteriegemeenschappen hingen wel met elkaar samen, maar reageerden heel verschillend op de gradiënt. Schimmels reageerden het sterkst en waren ook het sterkst gekoppeld aan de algehele verandering in bodemfunctioneren. Bacteriën reageerden niet duidelijk op de gradiënt zelf, al hingen ze wel samen met hoe breed het koolstofgebruik was. De hoeveelheid organische koolstof in de bodem liet van alle niet-levende eigenschappen de sterkste reactie zien: deze nam toe naarmate het beheer extensiever werd, en hing negatief samen met mestgebruik en het aantal dieren per hectare. Dat past bij het idee dat er bij minder intensief beheer meer plantenresten en wortelmateriaal in de bodem achterblijven. Omdat organische koolstof ook nauw samenhing met schimmelsamenstelling en afbraaksnelheid, vormen deze drie (organische koolstof, afbraaksnelheid, en schimmelsamenstelling) samen een betrouwbare indicator van hoe intensief een perceel op dit moment wordt beheerd in mijn studie.

De zuurgraad (pH) van de bodem vertelde een ander verhaal. Deze volgde niet de beheersintensiteit, maar leek vooral bepaald te worden doordat individuele boeren wel of niet besluiten om te bekalken, los van hoe intensief ze verder boeren. Wel bleek pH de sterkste voorspeller van enzymactiviteit, die zelf ook geen duidelijk verband hield met de gradiënt. Dit betekent dat je enzymactiviteit niet zomaar kunt gebruiken als eenvoudige graadmeter voor hoe intensief een perceel al jarenlang wordt beheerd.

Bodemfunctioneren meten: waarom samengestelde multifunctionaliteitsindices misleidend kunnen zijn

Dat de drie processen uit hoofdstuk 3 – die allemaal bijdragen aan dezelfde bodemfunctie, de kringloop van voedingsstoffen – zo verschillend reageerden, is belangrijk voor hoe

we bodemfunctioneren zouden moeten meten. In veel studies wordt namelijk een "multifunctionaliteitsindex" gemaakt: je neemt een paar processen die bij een bepaalde functie horen, en middelt die tot één cijfer. Ik betoog dat je daar voorzichtig mee moet zijn, ook als die processen bij dezelfde functie horen. Het is belangrijk om eerst te checken of die processen wel echt met elkaar meebewegen, voordat je ze samenvoegt tot één getal.

mVOCs als een mogelijke monitoringproxy

Microbiële vluchtige stoffen (mVOCs) zijn geurstoffen die bodembacteriën en -schimmels afgeven. Deze zijn onlangs voorgesteld als een manier om te meten wat de staat (activiteit en functioneren) van de microbiële bodemgemeenschap is. Hoofdstuk 4, 5 en 6 onderzochten dit vanuit drie kanten: reageren mVOCs op de beheersgradiënt, zijn ze bruikbaar in de praktijk, en hoe presteren ze in vergelijking met een al bestaande maat; enzymactiviteit.

In hoofdstuk 4 bleek het totale mVOC-profiel niet duidelijk te verschillen tussen de verschillende typen landgebruik, vooral omdat er binnen elk type al veel variatie was. Met een gerichte statistische methode (TITAN2) vond ik zestien specifieke geurstoffen die wél duidelijk meebewogen met de gradiënt. Net als in hoofdstuk 3 bleken schimmelsamenstelling (maar niet bacteriesamenstelling) en bodem-pH weer de belangrijkste factoren te zijn die samenhingen met het mVOC-profiel. Dit bevestigt dat deze twee in meerdere hoofdstukken een centrale rol spelen. Een andere bevinding: bekende koppelingen tussen specifieke geurstoffen en de microben die ze zouden produceren, gebaseerd op eerder laboratoriumonderzoek, bleken in dit veldonderzoek grotendeels niet aanwezig te zijn. Dat laat zien hoe sterk deze signalen afhangen van de context zodra je ze buiten het lab, in echte bodems, bestudeert.

In hoofdstuk 6 heb ik de 16 mVOCs uit hoofdstuk 4 rechtstreeks vergeleken met 9 enzymactiviteiten (uit hoofdstuk 3), een gangbare methode voor het meten van bodemfunctioneren. Eerst keek ik, zonder de percelen vooraf in te delen naar beheerscategorie, of er vanzelf duidelijke groepen zichtbaar werden in de data. Dat bleek niet het geval: geen van beide methoden liet duidelijke, vanzelf ontstane groepen zien tussen de typen landgebruik, en de patronen die de twee methoden lieten zien, leken ook niet erg op elkaar. Dit is niet per se een zwakte van beide methoden, maar wellicht een teken dat bodemfunctioneren simpelweg niet netjes past binnen de hokjes "gangbaar", "extensief" en "half-natuurlijk". Toch bleek er, ondanks dit verschil, een sterk gedeeld onderliggend patroon te zitten tussen mVOCs en enzymactiviteit. Bovendien liet ik het model dit keer wél vooraf weten tot welke beheerscategorie elk perceel behoorde, en vroeg ik het om die categorie te voorspellen op basis van de gemeten data. In deze gestuurde analyse bleken

mVOCs juist veel beter dan enzymactiviteit in staat om de beheerscategorie van een perceel correct te voorspellen; een resultaat dat ik zelf niet had verwacht. Dit past bij de bevinding uit hoofdstuk 3 dat enzymactiviteit, in tegenstelling tot schimmelsamenstelling, nauwelijks samenhing met de gradiënt.

Hoofdstuk 5 combineerde gesprekken met boeren met een workshop met bodemonderzoekers, om te achterhalen wat een bruikbare bodemsensor eigenlijk zou moeten kunnen. Vooral gangbare boeren waren geïnteresseerd: zij wilden een instrument dat kon laten zien of de aanpassingen die ze in hun beheer doorvoerden, ook echt effect hadden. Ze vonden praktische, vergelijkbare informatie (verschillen binnen één perceel, vroege waarschuwingssignalen, vergelijking met referentiewaarden) belangrijker dan een sensor die simpelweg bevestigt in welk hokje (gangbaar, extensief, biologisch) hun bedrijf valt. Onderzoekers kwamen tot een vergelijkbare conclusie: een bruikbare sensor moet de gemeten stoffen kunnen koppelen aan concrete bodemprocessen, in plaats van alleen een lijst met mVOCs af te leveren. Zij wezen wel op praktische obstakels, zoals het gebrek aan goede referentiedatabases.

Samen laten deze hoofdstukken zien dat mVOCs echt potentie hebben als brede indicator om te volgen of, en in welke richting, een bodem verandert. Om dat potentieel waar te maken is meer onderzoek nodig: op andere bodemtypen, met duidelijke referentiewaarden om metingen tegen af te zetten, en met herhaalde metingen over meerdere jaren in plaats van de eenmalige meting die in dit proefschrift is gebruikt.

Een gradiënt begrijpen betekent niet dat je hem kunt sturen

De laatste vraag; kun je de overgang naar extensiever beheer versnellen?, staat centraal in hoofdstuk 7. Hierin combineerde ik een kasexperiment met een tweejarige praktijkproef, waarbij ik grond van een kruidenrijk referentieperceel gebruikte om een akkerperceel mee te "enten" (inoculeren), die we daarna inzaaiden met verschillende types kruidenrijkgraszaad mengsels. Daarmee wilden we onderzoeken of we hiermee het ontwikkelen van een bepaald bodemleven in de ingezaaide akker konder sturen. In de kas werkte dit goed: het toevoegen van verse donorgrond stuurde zowel de bacterie- als de schimmelgemeenschap duidelijk richting die van de donorgrond. De keuze van het zaadmengsel deed er in de kas veel minder toe dan de inoculatie zelf, wat erop wijst dat het waarschijnlijk de levende bodemorganismen in de donorgrond waren die deze verandering veroorzaakten.

In het veld gebeurde er na twee jaar echter niets vergelijkbaars: ongeacht welk zaadmengsel was gebruikt, was er geen duidelijk effect van de inoculatie te zien. Wel verschoven de bodemgemeenschappen in zowel de geïnoculeerde als de niet-geïnoculeerde percelen langzaam richting die van de donorgrond, wat waarschijnlijk komt doordat het

perceel geleidelijk overging van akkerbouw naar grasland, los van de inoculatie. Ook in de specifieke functies van de bodemgemeenschap (wat de organismen dóén, niet alleen wie erin zit, op basis van DNA) was in geen van beide experimenten een duidelijk effect van inoculatie te zien.

Een paar dingen verklaren waarschijnlijk waarom het in het veld niet werkte terwijl het in de kas wel werkte: we gebruikten in het veld een dunne laag donorgrond (om het praktisch haalbaar te houden), de bodem was van tevoren misschien niet genoeg verstoord om ruimte te maken voor de nieuwe organismen, en twee jaar is mogelijk simpelweg te kort.

Kortom: bodeminoculatie met levende grond heeft duidelijk potentie om bodemgemeenschappen te sturen, zoals de kas liet zien, maar dat potentieel vertaalde zich niet naar een snel, meetbaar effect onder realistische omstandigheden in het veld. Dat betekent niet dat de methode geen kans heeft voor de praktijk, maar wel dat er waarschijnlijk meer tijd en aanvullende maatregelen nodig zijn, zoals een dikkere laag donorgrond of het van tevoren beter verstoren van de bodem.

De transdisciplinaire context

Ik sluit dit proefschrift af door de ecologische bevindingen te plaatsen in een bredere, maatschappelijke context. Weten wát er ondergronds verandert bij extensivering is niet hetzelfde als weten wat een boer nodig heeft, financieel of sociaal, om die overstap ook echt te maken. Certificeringssystemen en beleidsregels werken vaak nog steeds met vaste categorieën van beheer, terwijl dit proefschrift juist laat zien dat een gradiënt de werkelijkheid beter weerspiegelt. De gesprekken met boeren en andere betrokkenen zoals uit hoofdstuk 5, zijn een eerste, kleine stap naar instrumenten die wetenschappelijke inzichten kunnen koppelen aan de praktische, geleidelijke manier waarop boeren hun bedrijfsvoering daadwerkelijk aanpassen.

Overkoepelende conclusie

De rode draad van dit proefschrift is een andere manier van kijken: extensivering is geen knop die je omzet tussen twee categorieën, maar een geleidelijk proces. Dit proces speelt zich af in de bodem, via eigenschappen en gemeenschappen die allemaal in hun eigen tempo veranderen en door verschillende mechanismen worden gestuurd. Dit levert drie concrete inzichten op voor de bodems gemeten in deze studie. Ten eerste blijken organische koolstof, afbraaksnelheid en schimmelsamenstelling betrouwbare graadmeters te zijn voor hoe intensief een perceel op dit moment wordt beheerd. Ten tweede blijken mVOCs een veelbelovende, maar contextafhankelijke manier om bodems

te monitoren, vooral geschikt om geleidelijke verandering te volgen. Ten derde laat het inoculatie-experiment zien dat het begrijpen van hoe een gradiënt in elkaar zit, nog niet betekent dat je een perceel er ook snel langs kunt sturen. Samen pleiten deze bevindingen voor het monitoren van geleidelijke veranderingen met meerdere, contextgevoelige indicatoren, en voor beleid dat ruimte biedt aan geleidelijke, doorlopende overgangen in plaats van alleen aan vaste categorieën.



Photograph taken at the author's family farm in Zeeland, the Netherlands.

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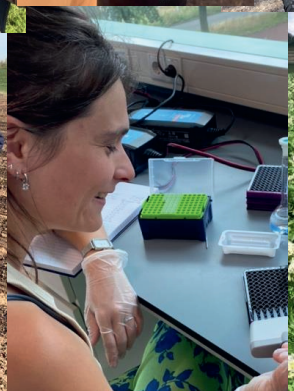










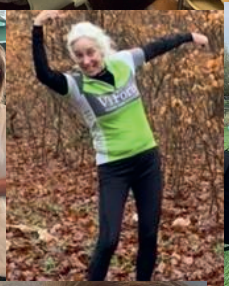








Utah



restaurant Giga-Bit










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About the author

Rosa was born on September 26, 1995, in Zutphen, and grew up in the village of Voorst, surrounded by dairy farmers along the fertile lands of the Ijssel. There she spent her youth going to "tentfeesten", celebrating carnaval, helping out in the garden, playing soccer in an all-girls team, and spending her summers in Zeeland. Her father grew up on a "gemengd" bedrijf (a mixed farm) in Zeeland, and the photographs appearing throughout this thesis were taken there. He witnessed for himself how the extensive family farm underwent intensification during the years. Spending many childhood summers in Zeeland, Rosa got her own early taste of farming life. Her father later ran a waste recycling company, from which she developed an early interest in compost (the subject of her group 8 presentation). The photographs in the thesis are a reference to her family in Zeeland. In secondary school she chose the "economie en maatschappij" track, and biology, was a subject she almost did not pass. It was in a geography class, however, watching the Zembla documentary "Groen Goud" on the restoration efforts on the Chinese Loess plateau, that she first knew she wanted to do something with nature restoration. Unable to settle on a single discipline, she enrolled in the interdisciplinary bachelor's programme Future Planet Studies at the University of Amsterdam. As her interest turned toward the idea that restoring ecosystems requires starting at the bottom (e.g. the soil), she followed the Earth Sciences track, which meant catching up on physics, chemistry and biology. She continued into a master's in Earth Sciences at the same university, where her fascination with soil life and its role in ecosystem restoration only grew. Her master's thesis took her to a glacier forefield in Switzerland, measuring soil organic carbon and glomalin (a sticky substance fungi produce) and analysing the terrain by drone. Her internship brought her to Tanzania with JustDiggit, studying carbon stocks and the effect of farmer-led natural regeneration on soil. Rosa had never intended to become a scientist, but a PhD felt more adventurous than the job descriptions she encountered. Coming from an entrepreneurial family, and having run her own small start-up (De Bierbakkers), she found in her PhD a similar kind of entrepreneurship. It bring together her love of the countryside, farmers, soil life, and novel technologies such as the mVOC sensor. She is now doing a postdoc within Curvebend, bending the curve of biodiversity loss through the lens of collective action. In her role, she

is responsible for laying the groundwork to integrate the project's three disciplines within the Dutch work package. She now lives in Haarlem, though she still finds her way back to Voorst, to the garden of her parents, and enjoying long runs in the country side. Besides the country side, she loves being in the mountains, especially in the winter.

Research Data Management

The research in this thesis has been carried out under the RDM policy of the Radboud Institute for Biological and Environmental Sciences, version 25-Mar-2026, accessed at `https://www.ru.nl/ribes`. All data used in this thesis is archived per chapter in online data repositories. Data can be accessed using below persistent identifiers:

Chapter 1	No data was used
Chapter 2	Data available from the DANS-EASY data repository: `https://doi.org/10.17026/LS/J4Y6FO`
Chapter 3	Data available from the Zenodo data repository: `https://doi.org/10.5281/zenodo.20539936`
Chapter 4	Data available from the DANS-EASY data repository: `https://doi.org/10.17026/LS/32VHKC`
Chapter 5	No data was used
Chapter 6	Data available from the Zenodo data repository: `https://doi.org/10.5281/zenodo.19451680`
Chapter 7	Data available from the Zenodo data repository: `https://doi.org/10.5281/zenodo.19454723`
Chapter 8	Data from Chapter 3 was used

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 30 ECTS (= 22 weeks of activities)

Review/project proposal
- PE&RC project proposal; Towards smart soil-sensing technology: assessing the effects of biodiversity on soil functionality in grasslands and arable fields | 4.5 ECTS

In-depth / Topical / On-site Postgraduate Courses
- Biogenic Volatiles - Exchange at Different Scales and Interactions with Ecosystem Processes | University of Copenhagen | 2021 | 1.5 ECTS
- Enzymatic activity assays | York University | 2023 | 1.5 ECTS

Methodological / Statistical Postgraduate Courses
- Soil Biology Lab Skills Course | Wageningen University | 2022 | 1.5 ECTS

Laboratory training and working visits
- VOC data processing | Copenhagen University | 2023 | 0.9 ECTS

Invited review of journal manuscripts
- Journal of applied ecology | Above- and belowground organisms coordinate soil micro-food webs to enhance soil multifunctionality | 2023 | 1 ECTS
- Nature | Pesticide residues in soils differentially affect soil biodiversity | 2025 | 1 ECTS

Competence, skills and career-oriented activities
- PE&RC writing week- January 2024 | PE&RC | 2024 | ECTS
- Oikos PhD workshop 'Visuals in Science' | Oikos, Lund | 2024 | 2 ECTS

Scientific Integrity/Ethics in science activities
- Ethics in Plant and Environmental Sciences | Wageningen University | 2025 | 0.3 ECTS

PE&RC Retreat, PE&RC Day, and other PE&RC events
- PE&RC First year retreat | 2022 | 1 ECTS

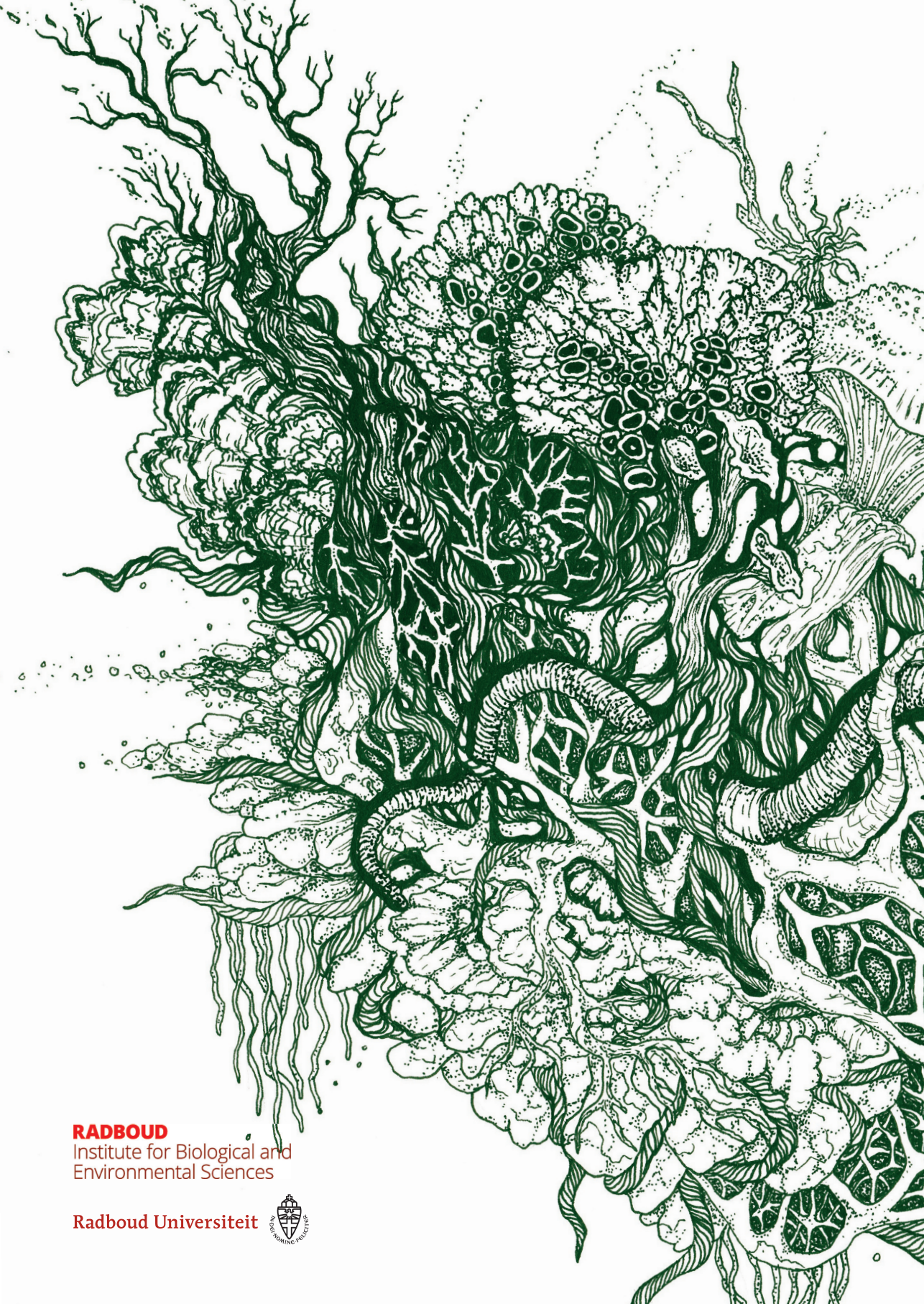
National/local scientific meetings, seminars, and discussion groups
- Emissions & Nutrients management | 2022-2023 | 3 ECTS
- Organising NCSE kick-off symposium | 2023 | 0.3 ECTS
- Organising and hosting 2nd NCSE symposium at Radboud University | 2024 | 0.6 ECTS
- NWO RC3 (talk) Explaining trends in biodiversity by scaling up from organism-environment relationships | 2024 | 0.3 ECTS

International symposia, workshops and conferences
- Global Soil Biodiversity conference (poster) | Dublin | 2023 | 1.9 ECTS
- VOLT symposium (talk) | Copenhagen | 2025 | 1.6 ECTS
- EGU (talk) | Vienna | 2025 | 2.5 ECTS
- Teabag Symposium (talk) | Online | 2023 | 1.3 ECTS
- Oikos (poster) | Lund | 2024 | 1.9 ECTS
- NAEM (talk) | Ede | 2024, 2026 | 3.2 ECTS
- NCSE (talk and Workshop) | 2024, 2025 | 2.6 ECTS
- Eindsymposium Living Lab Ooijpolder Groesbeek | Ede | 2026 | 1.3 ECTS

Societally relevant exposure
- Vroege Vogels radio interview Radio NPO1 | 2025 | 0.2 ECTS
- Trouw article | 2025 | 0.2 ECTS
- Weekend school excursie bodemzicht (primary school) | 2023-2024 | 0.4 ECTS
- BNR podcast Wetenschap vandaag "Kunnen bodemtransplantaties zorgen voor gezondere bodem?" | 2024 | 0.2 ECTS
- BNR podcast Wetenschap "Elektronische neus helpt bepalen hoe het met onze bodem gaat" | 2024 | 0.2 ECTS
- VOX podcast In a Nutshell ep. 4 | 2023 | 0.2 ECTS
- NWO film "Living Lab Ooijpolder Groesbeek" | 2026 | 0.2 ECTS
- Radboud podcast Science Snacks | 2023 | 0.1 ECTS
- Article Magazine Van Onze Grond | 2026 | 0.3 ECTS
- Article Magazine Landschap | 2025 | 0.1 ECTS
- Artikel vakblad NWO resultaat – "Uit de verstikking" | 2023 | 0.2 ECTS
- Blog ViaNatura "Ruiken of de bodem gezond is" | 2023 | 0.2 ECTS
- Development Course Ecology artistic Platform DIS | 2023-2024 | 1 ECTS
- Sit with a Scientist Video | 2024 | 0.3 ECTS
- Platform DIS "Programma Bodemleven" | 2022 | 1.4 ECTS
- Bodemdierentelling Radboud | 2023-2024 | 0.2 ECTS
- De Volkskrant "Experiment: stop schimmels in de grond om de droogte te weerstaan" | 2023 | 0.1 ECTS
- New years reception Soil Valley presentation | 2025 | 0.2 ECTS

Lecturing/Supervision of practicals/tutorials
- Management of Ecosystems (MoE) | 2024 | 5 | 0.8 ECTS
- GIS | 2023, 2024 | 14 days | 1.6 ECTS

BSc/MSc thesis supervision (5 ECTS)
- Research topic 1: SOC in agricultural grasslands (Bsc internship)
- Research topic 2: Respiration in agricultural grasslands (Msc internship)
- Research topic 3: Optimalisation of mVOC extraction (1) (HBO internship)
- Research topic 4: Optimalisation of mVOC extraction (1) (Msc internship)
- Research topic 5: Microbial inoculation for agriculture (Msc review)
- Research topic 6: Enzymatic activity in agricultural grasslands under climate change (Msc internship)
- Research topic 7: Bsc internship VOCs (not completed)
- Research topic 8: OpenED Msc student VOCs for sensor application (Msc internship)
- Research topic 9/10: Open ED Aeres students (2) 2023 (HBO internship)
- Research topic 11/12: Open ED Aeres students (2) 2024 (HBO internship)
- Research topic 13: Msc internship inoculation (to be finished)
- Research topic 14: ACT project (8 students, WUR)



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