Composition of microbiomes along the trophic chain in agricultural grassland ecosystems

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Nothing in life is to be feared,

it's only to be understood.

- Marie Curie

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Summary

Grassland ecosystems are hubs of biodiversity and as being one of the most common ecosystem types in central Europe they play a key role in agricultural land use. As pastures and meadows, grassland ecosystems are increasingly subject to intensified land use including high frequencies of mowing, grazing and fertilization. Worryingly, such intensive land use is one of the major drivers of biodiversity loss. As major land use component, fertilization, including organic fertilization with animal manure, may have tremendous impacts on the ecosystem including all organisms. Microbes, as the smallest members of an ecosystem, play essential roles in the health of their host and the environment with soil microbiomes contributing to important ecosystem functions. Land use and especially fertilization have been shown to impact microbial communities by changing community composition and diversity of microbiomes. So far, such effects have only been studied in isolated compartments and mainly after long-term application of fertilizers. However, within an ecosystem, trophic compartments and their associated microbial communities are in constant interaction creating a network of ecosystem functions and services. These interactions need to be taken into account when assessing the impacts of anthropogenic management in grassland ecosystems. In this thesis I present a comprehensive overview on the compositions of microbial communities along the grassland trophic chain starting from the soil and including plants and animals to assess the effects of different organic fertilizers and the associated fertilization history acting on them.

Chapter 1 of this thesis represents a literature review on different biotic and abiotic factors impacting soil microbiomes and the role of agricultural practices in shaping soil microbial communities. Besides pH, soil moisture and temperature, changes in the nutrient composition as well as the introduction of allochthonous bacteria, carrying different genetic makeup, change the composition and diversity of soil microbial communities. As such, agricultural practices play a key role in impacting soil microbiomes and subsequently also the associated ecosystem functions including nutrient cycling, organic matter decomposition, plant growth promotion or supporting abiotic stress tolerance. In chapter 2, the field study of microbial communities associated to different compartments along the trophic chain including soil, roots (i.e., rhizosphere and endosphere) and flowers of the legume *Trifolium pratense*, earthworms, voles and bumblebees on agricultural grassland revealed strong impacts of different organic fertilizers on all microbial communities along the trophic chain. These effects of fertilization with biogas digestate, cow and horse manure and pig slurry fertilization on microbial communities were different in above-and belowground compartments and demonstrated distinct host-dependent shifts with pig slurry fertilization imposing the most significant effects. Overlapping genera between compartments suggest that trophic interactions among these different hosts might cause cascading effects of fertilization induced changes to the microbial communities

from lower trophic compartments to higher trophic compartments underlining the need for studies including several hosts. Based on the observations from the field study, chapter 3 aimed at recreating the grassland ecosystem and the observed legacy effects of long-term pig slurry fertilization history on microbial communities along the trophic chain in a controlled lab-scale mesocosm set up. The comparison of established mesocosms with a history of repeated pig slurry application to long-term fertilized field samples revealed strong legacy effects of long-term pig slurry application outcompeting the effects of individual application in soil and flowers, while rhizosphere and endosphere reflected additional impacts of short-term fertilization. Overlaps of important bacterial genera between field and mesocosm samples and similar effects on the different trophic compartments suggest that mesocosm set ups can recreate key ecosystem interactions of grassland ecosystems and partially simulate long-term fertilization effects on microbial communities in a controlled set up.

The studies presented in this thesis shed light on the impact of agricultural land use, especially organic fertilization and the application history, on microbial communities along the trophic chain. By studying interconnected microbiomes in the field and in controlled lab-scale mesocosms, I could show, that different organic fertilizers and different histories of pig slurry fertilization do not act in the same way on above and belowground compartments of the trophic chain extending the knowledge on soil microbiomes summarized in chapter 1. By following the eco-holobiont frame work, which integrates interactions among microbial communities and the network of microbiomes essential for ecosystem functioning, the findings of chapter 2 and 3 underscore the need for comprehensive cross-scale approaches assessing different microbial communities across different time scales. Understanding the full impact of fertilization on different compartments along the trophic chain within an ecosystem is essential to establish measures for a sustainable agriculture securing food production and, within the One Health concept, assuring ecosystem health including plants and animals and ultimately sustaining human health and wellbeing.

Deutsche Zusammenfassung

Grünland-Ökosysteme sind nicht nur Hotspots der Biodiversität, sondern spielen als eines der am weitest verbreiteten Ökosysteme in Zentraleuropa auch eine Schlüsselrolle in der Landwirtschaft. Durch die Nutzung als Weiden und Wiesen zur Heu- und Silage-Produktion, sind Grünland-Ökosysteme einer immer intensiver werdenden Landnutzung einschließlich Beweidung, intensiver Düngung und Mahd ausgesetzt. Beunruhigender Weise ist diese intensive Landnutzung einer der Hauptfaktoren für den Verlust von Biodiversität in Grünland-Ökosystemen. Hierbei haben organische Düngemittel, wie Dung oder Gülle, einen signifikanten Einfluss auf das Ökosystem und dessen Bestandteile. Mikroorganismen, als die kleinsten Mitglieder eines Ökosystems, spielen eine wesentliche Rolle für die Gesundheit ihres Wirtes. Die Zusammensetzung, und Diversität der mikrobiellen Gemeinschaften wird jedoch stark durch intensive Landnutzung, insbesondere Düngung, beeinflusst. Bislang wurden solche Effekte hauptsächlich in isolierten Kompartimenten und nach langfristiger Düngung untersucht. Innerhalb eines Ökosystems stehen jedoch nicht nur verschiedene trophische Kompartimente, sondern auch die dazugehörigen Mikrobiome in ständiger Wechselwirkung und bilden ein Netzwerk an Ökosystemfunktionen und -dienstleistungen. Bei der Analyse der Auswirkungen anthropogener Landnutzung auf Grünlandökosysteme, ist es daher von entscheidender Bedeutung, die engen Wechselwirkungen zwischen Wirten zu berücksichtigen und mehrere Kompartimente zu untersuchen. Die vorliegende Dissertation gibt einen umfassenden Überblick über die Zusammensetzungen verschiedener Mikrobiome entlang der Grünland-Nahrungskette beginnend beim Boden über die Pflanze bis hin zu Bestäubern, Detritivoren und Herbivoren und legt die Auswirkungen, die verschiedene organische Dünger und deren Anwendungshistorie auf die mikrobiellen Gemeinschaften haben, dar.

Kapitel 1 dieser Dissertation gibt einen Überblick über den aktuellen Stand der Forschung zu biotischen und abiotischen Faktoren, welche das Bodenmikrobiom beeinflussen, und ordnet den Einfluss landwirtschaftlicher Nutzung auf die Zusammensetzung mikrobieller Gemeinschaft im Boden ein. Neben abiotischen Parametern wie dem pH-Wert, der Bodenfeuchtigkeit und -temperatur, wirken sowohl Veränderungen in der Nährstoffzusammensetzung, als auch die Einführung allochthoner, düngerassoziierter Bakterien mit unterschiedlicher genetischer Ausstattung, auf die Zusammensetzung und Diversität der mikrobiellen Gemeinschaft. Landwirtschaftliches Management hat damit bedeutenden Einfluss auf die Dynamik des Bodenmikrobioms und die damit verbundenen Ökosystemfunktionen, wie die Steuerung biogeochemischer Nährstoffkreisläufe, die Zersetzung von organischer Substanz, die Förderung von Pflanzenwachstum sowie die Unterstützung der Toleranz von Pflanzen gegenüber abiotischem Stress. Auf diesen Wissensstand aufbauend wurde in Kapitel 2 durch eine großangelegte Feldstudie auf landwirtschaftlich genutzten Grünflächen und der Beprobung von

Boden, Wurzeln (Rhizosphäre and Endosphäre) und Blüten der Leguminose *Trifolium pratense*, Regenwürmern, Wühlmäusen sowie Hummeln, ein starker Einfluss von organischer Düngung auf alle assoziierten Mikrobiome nachgewiesen. Biogasgärreste, Rinder- und Pferdemist sowie Schweinegülle wirkten unterschiedlich auf oberirdische und unterirdische Kompartimente der Nahrungskette und wiesen stark wirtsspezifische Effekte auf, wobei die Düngung mit Schweinegülle die stärksten Veränderungen bewirkte. Der Nachweis überlappender bakterieller Genera zwischen den verschiedenen Kompartimenten deutet hierbei darauf hin, dass trophische Wechselwirkungen zwischen den einzelnen Kompartimenten zu kaskadenartigen Effekten führen könnten. Die durch die Düngung verursachten Veränderungen könnten sich somit von niedrigeren zu höheren trophischen Kompartimenten übertragen. Dies unterstreicht erneut die Notwendigkeit eines umfassenden und integrativen Ansatzes. Basierend auf den Ergebnissen dieser Feldstudie, zielt Kapitel 3 darauf ab, ein Grünland Ökosystem im kontrollierten Labor-Maßstab in Mesokosmen nach zu bauen und die beobachteten Langzeiteffekte von Schweinegülle auf mikrobielle Gemeinschaften entlang der Nahrungskette zu reproduzieren. Mesokosmen stellten hierbei im Vergleich zu langzeit- gedüngten Feldflächen, eine kurze Historie von wiederholter Düngung mit Schweinegülle dar. Der Vergleich von Mesokosmen und Feldflächen ergab starke Langzeitwirkungen von Schweinegülle-Düngung, welche die kurzzeitigen Effekte einzelner Dünge-Applikationen in Boden und Blüten überwogen. Beide Wurzelkompartimente (Rhizosphäre und Endosphäre) wiesen hingegen zusätzliche Kurzzeiteffekte einzelner Düngungen auf. Überlappende bakterielle Genera zwischen Feld und Mesokosmen, sowie ähnliche Effekte innerhalb einzelner Kompartimente, deuten darauf hin, dass die in Kapitel 3 etablierten Mesokosmen in der Lage sind, wichtige ökologische Wechselwirkungen von Grünlandökosystemen nachzubilden und langfristige Düngungseffekte auf die unterschiedlichen mikrobiellen Gemeinschaften in einer kontrollierten Umgebung zumindest teilweise zu simulieren.

Die in dieser Dissertation zusammengefassten Arbeiten beleuchten den Einfluss landwirtschaftlicher Nutzung, insbesondere der organischen Düngung und deren Anwendungshistorie, auf mikrobielle Gemeinschaften entlang der Nahrungskette. Durch die Untersuchung miteinander vernetzter Mikrobiome, konnte ich zeigen, dass verschiedene organische Düngemittel und unterschiedliche Anwendungshistorien von Schweinegülle sehr wirtsspezifisch und nicht in gleicher Weise auf oberirdische und unterirdische Kompartimente der Nahrungskette wirken. Damit erweitern Kapitel 2 und 3 die in Kapitel 1 zusammengefassten Effekte und verdeutlichen die Notwendigkeit eines umfassenden Ansatzes zur Bewertung des Einflusses landwirtschaftlichen Managements auf unterschiedliche mikrobielle Gemeinschaften. Basierend auf dem Konzept des Öko-holobionts, welches die Gemeinschaft von Mikrobiomen verschiedener Organismen berücksichtigt, sind Interaktionen und Wechselwirkungen zwischen Wirten und ihren Mikrobiomen der Schlüssel zu einem

mikrobiellen Netzwerk, welches essentiell für diverse Ökosystemfunktionen ist. Ein allumfassendes Verständnis der Auswirkungen von organischer Düngung auf unterschiedliche Kompartimente der Nahrungskette im Grünland ist dementsprechend unerlässlich für die Etablierung von Maßnahmen für eine nachhaltige Landwirtschaft und die Sicherstellung der Nahrungsmittelproduktion. Dies sichert im Rahmen des One Health Ansatzes nicht nur die Gesundheit des Ökosystems einschließlich aller Pflanzen und Tiere, sondern letztlich auch die Gesundheit und das Wohlbefinden der Menschen.

General introduction

Grassland ecosystems are hubs of biodiversity

Grasslands are one of the most common ecosystem types in Europe and worldwide at present covering about 40% of earth's surface (Petermann & Buzhdygan, 2021). As such, they have been subjected to long-term human activities since the beginning of agricultural practices during the Mesolithic-Neolithic transition (Hejcman et al., 2013). Ancient low-intensity management including practices including-mowing and grazing resulted in semi-natural open grasslands, which created habitats for diverse plant species (Karlík & Poschlod, 2009; Pärtel et al., 2007). Today, semi-natural grasslands are among the most diverse ecosystem types in Europe (Habel et al., 2013). As such they keep the record of plant species diversity on small scale (Wilson et al., 2012) and harbor diverse communities of above and belowground living animals as well as different microbial and fungal communities (Bardgett & Cook, 1998; Petermann & Buzhdygan, 2021; Wilson et al., 2012). In doing so, they shelter macro- and microorganisms that perform diverse functions in ecosystem services, contributing to nutrient acquisition and cycling (e.g., nitrification, organic carbon cycling and phosphorous retention), soil aggregate stability, above and belowground plant biomass production, climate regulation, pathogen resistance and pest control (Soliveres et al., 2016). Moreover, grasslands ecosystems are key to ecological processes like pollination and water regulation and have high agricultural value in securing food production (Lindborg et al., 2023). Based on these diverse functions performed by different ecosystem members, high multitrophic diversity, i.e., high diversity of species across above- and belowground compartments, is essential to ecosystem functionality (Buzhdygan et al., 2020; Soliveres et al., 2016). To maintain ecosystem processes it should therefore be of high priority to conserve diverse grassland ecosystems and limit external disturbances.

Land use practices in grassland ecosystems

In addition to their great importance as biodiversity hubs, grasslands play a major role in agriculture. Growing demand for food and fodder for cattle and livestock, requesting higher nutritional value and higher biomass production, leads to more intensive land use and might transform semi-natural grasslands into less diverse and intensified grasslands (Allan et al., 2015; Gossner et al., 2016; Lindborg et al., 2023). Land use in grassland ecosystems can be categorized into three major components including grazing, mowing and fertilization.

As pastures, grasslands provide forage for grazing livestock like cows, sheep or horses which selectively reduce plant biomass, fertilize the sites and alter the soil structure through their movement. As meadows, grassland ecosystems are typically subject to frequent mowing to produce hay and silage for livestock feed during the winter months. Within grasslands, legumes represent a

key forage component for livestock due to their high protein and fiber contents, (Marshall et al., 2017) which enhances fodder quality and its nutritional value. Beyond their role in livestock feeding, legumes naturally enhance soil fertility through symbiosis with nitrogen fixing bacteria, (Fustec et al., 2010; Huss-Danell & Chaia, 2005), promote soil health and control weed growth (McKenna et al., 2018). Thus, integrating forage legumes into cropping systems has a long cultivation history in temperate regions and remains a central component of sustainable livestock farming (De Vega et al., 2015; Kjærgaard, 2003).

To further promote biomass production in grassland ecosystems, the addition of nutrients via fertilization is central to intensive land use. Nitrogen, phosphorous and potassium represent the key macronutrients limiting plant growth (Shrivastav et al., 2020; White & Brown, 2010) and, by applying inorganic as well as organic fertilizers, soils are supplement with these necessary nutrients (He et al., 2022; Shi et al., 2024). The development of the Haber-Bosch method facilitated large-scale ammonium synthesis, driving the widespread use of synthetic fertilizers in the last century. Nevertheless, the widespread availability and comparatively lower negative impact on plant diversity and organic carbon content, make originally used organic fertilizers like animal manure and digestates an attractive alternative (De Vries et al., 2015; He et al., 2022; Shi et al., 2024; Villa-Galaviz et al., 2023). In practice, livestock manure or slurry is either directly applied to the grassland sites, aerobically composted prior to application, or anaerobically digested in biogas plants with the resultant digestate used as fertilizer. Although the use of organic fertilizers is essential in enhancing biomass production, the associated changes in nutrient composition directly impact soil properties, like soil pH (L. Wu et al., 2020) and influence microbial communities.

Importance of microbial communities for host health and ecosystem functions

Just like humans, animals and plants and the environment rely on a myriad of microbial communities that perform important functions supporting host and ecosystem health (Barko et al., 2018; Engel et al., 2016; Hartmann & Six, 2023; Trivedi et al., 2020). Soil microbiomes are the basis of the grassland ecosystem and harbor very heterogeneous and diverse microbial communities (Xiong et al., 2021). These communities serve key ecosystem functions including nutrient cycling, organic matter decomposition, pollutant degradation, pest and disease control as well as climate regulation via impacting carbon fluxes (Gougoulias et al., 2014; Hartmann & Six, 2023; Van Der Heijden et al., 2008). Soil microbiomes further act as reservoir from which plants recruit beneficial microbes into the rhizosphere through rhizodeposition of various sugars, organic acids, amino acids and secondary metabolites (e.g., phenolics) (C. X. Yang et al., 2025). This nutrient-rich environment creates a competitive habitat harboring a distinct microbial community (Reinhold-Hurek et al., 2015) which is

considered the plant's second genome extending its functional diversity and conferring adaptive benefits (Berendsen et al., 2012; Mendes et al., 2013). By colonizing the rhizosphere (i.e., the soil adherent to the root) and the endosphere (i.e., the tissue within the root), microbes directly promote plant growth by nutrient fixation or production of phytohormones and plant growth promoting factors. They build the first line of defense against invading pathogens (Berg et al., 2020; H. Liu et al., 2021; Trivedi et al., 2020) and under abiotic stress like drought, flooding, salinity or heavy metal pollution, plants rely on their root microbiomes for enhanced stress resilience ensuring plant health (J. Li et al., 2025).

Similar to root microbiomes, gut microbial communities in animals are essential for host health (Moran et al., 2019). These communities are interlinked to the immune system (Horak et al., 2020; Zheng et al., 2020), competitively inhibit pathogens and produce antifungal metabolites protecting their host against various infections (Miller et al., 2021; Steele et al., 2021). Beyond their role in host-protection against diseases, gut microbiomes are crucial in digestion and nutrient acquisition with specialized core members supporting the hosts in metabolizing diverse diets and adapting to their respective environment. For example, in pollinators, gut microbes facilitate the metabolism of complex carbohydrates and the degradation of pollen compounds (Lee et al., 2015, 2018) while microbial communities of detritivores (such as earthworms) support host adaptation by regulating cellulase activity (Y. Yang et al., 2023). As such, a host and its associated microbial communities have been described as an ecological unit, forming the holobiont where the microbiome expands the genetic and functional capacity of the host (Bordenstein & Theis, 2015).

Similar to macro-communities, microbial communities are sensitive to alteration in their environment. Disturbances in the homeostasis of a microbial community (i.e., a dysbiosis) caused by external factors can provoke the loss of commensal bacteria, potentially allowing proliferation of pathogens leading to severe consequences for host health (González & Elena, 2021; Steele et al., 2021). Given that various organisms contribute to diverse ecosystem functions, reduced host health can ultimately impact environmental health. Thus, exogenous disturbances compromising host health, e.g., pollinator health, might diminish pollination services and consequently cause a serious threat to ecosystem functioning (Maes et al., 2016). Likewise, alterations in soil microbial diversity and composition might affect important ecosystem functions like decomposition, nutrient retention and belowground carbon allocation (Wagg et al., 2014, 2019) which in turn can propagate across the ecosystem threatening its balance and overall functioning.

Impact of land use on microbial communities in grassland ecosystems

Intensive land use is one of the main drivers of biodiversity loss in grassland ecosystems with differential impacts on aboveground and belowground compartments (Aslani et al., 2024; Gossner et al., 2016; Le Provost et al., 2021). As it builds the basis of the grassland ecosystem, soil is affected by several land use practices. The use of heavy machinery on grassland sites compacts soil structure disrupting pore connectivity, water drainage and oxygen supply (Schäffer et al., 2008) and negatively impacts soil microbial communities (Longepierre et al., 2021). Additionally, fertilization with animal manure or slurry alters soil pH and reshapes soil and rhizosphere microbiomes (Schöps et al., 2018), but also introduces livestock associated microbes. These allochthonous microbes may displace indigenous members of the microbiome (Y. Yu et al., 2024) or introduce risk factors like antimicrobial resistance genes which can be transferred to the native community and spread in the environment (Götz & Smalla, 1997; Smalla et al., 2000). Alternatively, nutrient addition drives ecosystem-wide effects by primarily increasing plant biomass while simultaneously altering microbial communities across different trophic compartments. In soil, higher nutrient availability increases microbial biomass (Luo et al., 2015), but at the same time differentially modifies microbial diversity and composition (Bebber & Richards, 2022; Kong et al., 2023; Mora-Salguero et al., 2025). Subsequently, this reduces network complexity in both soil and roots (Ma et al., 2021; Romdhane et al., 2022; Schöps et al., 2018) and affects soil and root enzymatic activity (Guo et al., 2011; Schöps et al., 2018). Aboveground, flowers and leaves similarly experience reductions in diversity due to a loss of rare taxa, homogenization of the composition and reduced network complexity (Gaube et al., 2020). Thus, while fertilization enhances plant growth, it significantly affects microbial diversity and composition in multiple compartments of grassland ecosystems.

Agricultural grassland sites are typically managed over years and thus subject to a history of land use management. Accordingly, research has largely focused on long term-studies of land use factors with abovementioned effects of fertilization on microbial communities reflecting legacy effects. However, fertilization, which changes the nutrient profile and introduced allochthonous bacteria, might need a more stringent surveillance as individual fertilization events can also impose short-term effects on microbial communities. For instance, soil microbial community structure, functional potential as well as enzymatic activities have been shown to differ upon short-term fertilization (Guo et al., 2011; Kraut-Cohen et al., 2023; Lazcano et al., 2013). These short-term effects may reflect a temporal flexibility of soil microbial communities, for instance fertilization induced reduced diversity was found to recover to its initial status a few days after pig slurry fertilization (Suleiman et al., 2016) and manure associated taxa were no longer detectable 10 weeks after application (Tyrrell et al., 2023). Thus, ecological effects of fertilization seem to depend on the temporal scales of application underscoring the need for

combined short- and long-term studies to fully understand the consequences of fertilization on different microbial communities and their roles in the environment.

Interconnectedness of microbial communities along the trophic chain

Within ecosystems, microbial communities are not isolated, but interconnected through trophic interactions. These interactions link microbial communities across different hosts (Gralka et al., 2020; Thakur & Geisen, 2019; van Bruggen et al., 2019) and integrate food webs of macro-and microorganisms (Robinson et al., 2024) ensuring ecosystem functioning. Even though microbial compositions vary among different hosts, plants and animal guts share key bacterial taxa and functional traits (Mendes & Raaijmakers, 2015) with the microbiome of an individual host representing a subset of the broader environment (Pennisi, 2019). Along a trophic chain, i.e., a linear network of trophic interactions, soil microbiomes form the basis and interact with nearly all ecosystem compartments (H. Liu et al., 2019; Singh et al., 2023). They not only act as reservoirs from which plants actively recruit microbes into the direct root surrounding, i.e., the rhizosphere (H. Liu et al., 2021; Mendes et al., 2013), but they are also ingested by different animals (Mahaney & Krishnamani, 2003) and source aboveground phyllosphere microbiomes of leaves and flowers (Massoni et al., 2021). In this way, plants connect aboveground and belowground compartments by hosting distinct microbial communities in the roots and phyllosphere (Wolfgang et al., 2023). Phyllosphere associated microbial communities are shared along the plant axes between plant leaves, stem and flowers (Bai et al., 2015; Zhou et al., 2021), and interconnect with pollinators. Insect-pollinated flowers are reservoirs for microbes (McFrederick et al., 2017) and, due to frequent pollinator interactions, act as hubs for microbial transmission (Keller et al., 2021; Vannette, 2020). Pollinators, in turn, transport microbes between flowers, their colony members and across the landscape, thereby facilitating long-distance dispersal. In detritivores, such as earthworms feeding on soil, plant litter or dead root material, the gut microbial community has been shown to be composed of microbial communities they ingest (D. Liu et al., 2018). Similarly, herbivores feeding on roots and shoots tend to acquire root and phyllosphere associated microbial communities (L. Zhu et al., 2021). Even though these interactions among macro-organisms along a trophic chain are often unidirectional, microbial interconnectedness is bidirectional. Herbivores induce plant immune responses when feeding on it, while grazing animals and plants reintroduce microbes into the environment through feces and litter deposition, all affecting soil microbial communities (H. Jiao et al., 2025; Wolfgang et al., 2023; Xun et al., 2018). Together, these interactions close the loop of microbial communities linking soil, plants, pollinators, detritivores and herbivores into a dynamic and interconnected microbial network (H. Liu et al., 2019).

In light of these trophic interactions, the framework of the holobiont – considering the host and its microbiome as a functional unit - is insufficient to explain variation within a hosts microbial community composition. (Banerjee & van der Heijden, 2023; Bordenstein & Theis, 2015). Extending the holobiont concept, the eco-holobiont concept incorporates not only the host, but also the microbiomes of all interacting organisms within an ecosystem, thereby accounting for feedbacks from biotic and abiotic interactions and incorporating the ecological loop of microbes (H. Liu et al., 2019; Singh et al., 2020). Alongside, the One Health concept emphasizes that human health is strongly interlinked to ecosystem health including plant and animal hosts as well as abiotic compartments such as soil. Recognizing these concepts and the interconnectedness within the loop of microbial communities, it is necessary to study anthropogenic management like land use and fertilization in a comprehensive ecosystem-wide context to fully capture their impact on host, ecosystem and human health (Banerjee & van der Heijden, 2023; H. Liu et al., 2019; Singh et al., 2020; Y. G. Zhu et al., 2023)

Aims of this thesis

Grassland ecosystems are of critical importance for agriculture and biodiversity and have therefore been the focus of study for decades. Nevertheless, intensified land use of grasslands and its impact on different communities of organisms have raised concern about ecosystem health, putting land use management under increasing surveillance. Given the tight network of macro- and microorganisms, along with the interconnected microbial ecological loop, it is essential to investigate different, interacting compartments along the trophic chain to fully understand how cascading effects of land use practices propagate through the ecosystem (Banerjee & van der Heijden, 2023; H. Liu et al., 2019; van Bruggen et al., 2019). While previous studies demonstrated effects of fertilization on individual compartments or limited trophic interactions (Allan et al., 2015; Gossner et al., 2016; Le Provost et al., 2021), interactions among microbial communities have largely been overlooked. Furthermore, integrative approaches assessing the impacts of fertilization across abiotic, plant-associated as well as animal-associated microbial communities are still lacking. In order to address this knowledge gap, this thesis investigates the diverse impacts of short- and long-term application of organic fertilizers on different trophic compartments within agricultural grassland ecosystems, combining field studies and controlled laboratory experiments and using the microbiome as a marker for host and environmental health.

Given that soil and plants build the center of the microbial loop, the three chapters of this thesis focuses on soil and plant-associated microbial communities interacting with different animals (Figure 1). *Trifolium pratense L.* (red clover), a forage legume crop and a common member of temperate grassland ecosystems, was selected as target plant species. Owing to the symbiosis with nitrogen fixing

bacteria (Fustec et al., 2010; Huss-Danell & Chaia, 2005), *T. pratense* contributes to soil fertility and ecosystem health. Additionally, its high nutritional value makes it an important component of sustainable livestock farming (De Vega et al., 2015). Furthermore, by serving as food source for several pollinators and herbivores (Palmer-Jones et al., 1966; Schlegl-Bechtold, 1980; Stenseth et al., 1977), *T. pratense* links above- and below-ground compartments in a widely distributed trophic chain establishing its role as a keystone species in grassland ecosystems. These properties make it a suitable model organism for studying the effects of fertilization on microbial communities across trophic levels.

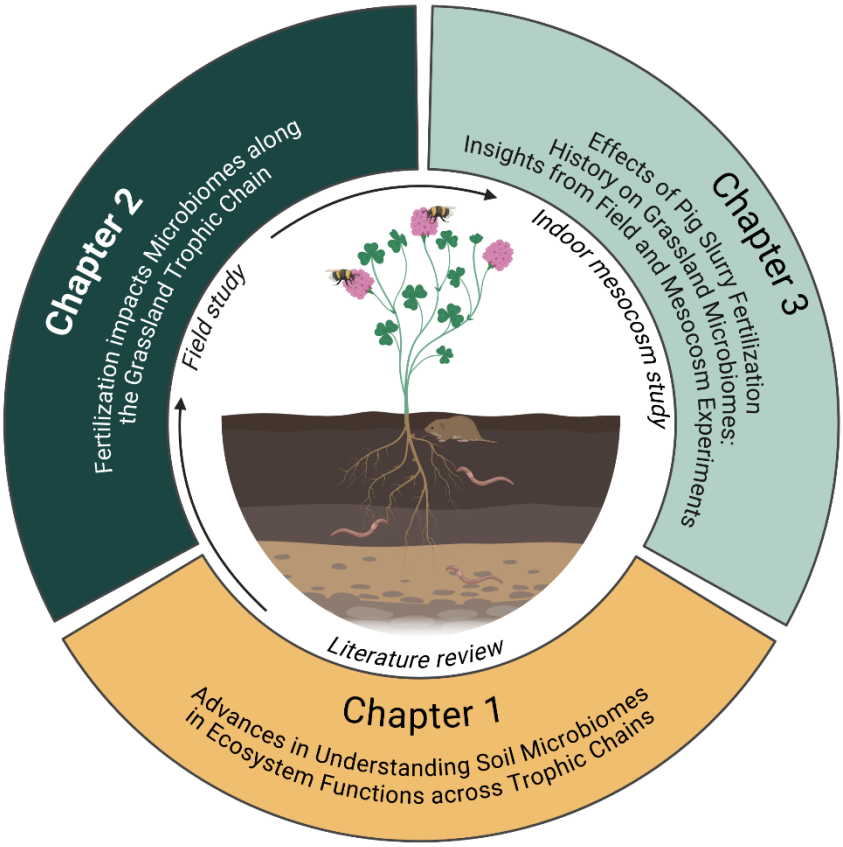

Figure1: Overview of the chapters of this thesis. Figure created with BioRender.com.

The first chapter of this thesis aims to establish the current state of knowledge on soil microbial communities building the base of a grassland ecosystem. In a review book chapter, I compiled the current literature on soil microbiomes, the factors impacting the community composition and diversity as well as the role of soil microbiomes in soil-plant interactions and in ecosystem functions. In doing so I asked the following questions: 1) What are the major factors impacting soil microbial communities and what role does land use play in it? 2) Which ecosystem functions are mediated by soil microbial communities and how important are interactions with other trophic compartments?

Building on the insights gained from the literature review, chapter 2 aimed to assess the impact of organic fertilization on microbial communities across the trophic chain by conducting a field study on the Swabian Alb. Samples were collected from seven different trophic compartments including soil, plant compartments (rhizosphere, root endosphere and flowers of *Trifolium pratense*), earthworm and vole feces, as well as bumblebee guts. Sampling sites included unfertilized sites as well as sites fertilized with either biogas digestate, cow or horse manure or pig slurry. The study was conducted within the frame work of the Biodiversity Exploratories (www.biodiversity-exploratories.de, funded by the German Research Foundation, DFG, (Fischer et al., 2010)) where the grassland sites under study are managed by local farmers and subjected to different long-term local land use practices. Control plots were located on a former military training site which cannot be managed intensively with heavy machinery. Here, only extensive grazing by free-roaming sheep provides low-intensity management making them ideal references. Comparing the control plots to plots fertilized with cow or horse manure, biogas digestate or pig slurry I asked 3) How do different organic fertilizers impact microbial communities across trophic compartments and do below-ground and above-ground compartments experience the same effects? 4) Are changes within the microbial communities driven by direct introduction of fertilizer associated microbes, or by fertilizer associated nutrient inputs?

Based on the results of chapter 2, I chose the most intensive fertilization i.e., pig slurry fertilization to differentiate between long- and short-term effects of fertilization. To do so, indoor mesocosm experiments were established by rebuilding a simplified grassland ecosystem under controlled laboratory conditions. Recreating a trophic chain, the set ups included soil, *Trifolium pratense* plants, earthworms and bumblebees. Fertilizing previously unfertilized soil with pig slurry created a history of repeated fertilization and short-term fertilization effects were assessed by sampling before and after a fertilization event. Legacy effects of long-term fertilization history were gauged based on samples collected on abovementioned field sites. By comparing mesocosm data with field results, this chapter aims to answer 5) Does intensive fertilization immediately alter microbial communities associated to different compartments after short-term application or are effects rather the result of long-term history of fertilization? 6) Can mesocosms mimic both natural ecosystems and microbial communities and, can they be used to simulate anthropogenic effects within a controlled environment?

Summary of chapters

Chapter1: Advances in understanding soil microbiomes in ecosystem functions across trophic chains.

Soil forms the basis of grassland ecosystems and provides essential functions for food production, with soil microbial communities being responsible for important ecosystem functions, e.g., decomposition of organic matter or nutrient sequestration and turnover that are fundamental to the soil biogeochemical cycling. Different factors impact soil microbiomes at the same time affecting its universal functions and. In this first chapter, I summarize the effects of different abiotic, climatic and land use associated factors on soil microbiomes and the role of soil microbial communities in ecosystem functioning via plant-soil interactions. Climatic factors, i.e., precipitation, temperature and drought as well as the combination of them mainly drive soil microbial communities by affecting soil moisture. Future climatic conditions with increasing temperatures and drought in combination with more extreme weather events, may threaten soil microbial communities. In addition, intensification of land usage in an agricultural context poses additional challenges to soil associated microbes. Soil compaction due to heavy machinery causes changes in soil density and structure, pore connectivity and water drainage and consequently impacts fungal hyphal and microbial networks. In line with that, fertilization mediated changes in soil macro- and micronutrients followed by associated changes in the soil pH, as well as input of allochthonous communities and their genetic material including antimicrobial resistance genes affect soil microbial diversity and community structures. Furthermore, soil and plant microbial communities are deeply interconnected as plants selectively recruit microbes from soil to their rhizosphere by rhizodeposition making soil microbiomes essential to plant fitness. These microbes then support plant growth and fitness either by producing plant growth promoting factors or by reallocating nutrients (e.g., nitrogen fixation in legumes), supporting nutrient uptake and promoting stress tolerance against pathogens and herbivores. Based on the One Health concept, human well-being is connected to the health of an ecosystem and its biotic and abiotic environment. Therefore, it is essential to understand interactions of soil with other compartments within the ecosystem and the different stressors that might threaten soil microbial communities and associated ecosystem services, in order to secure and sustain human, animal, plant and environmental health.

Chapter2: Fertilization impacts microbiomes along the grassland trophic chain

Intensive land use and fertilization in agricultural grassland ecosystems affect communities on the level of macro-organisms but have at the same time also a strong impact on microbial communities. Due to the interconnectedness of different compartments within a trophic chain of an ecosystem, a dysbiosis within one host might cause shifts in its interconnected compartments. However, studies assessing the impact of fertilization on diverse connected microbial communities are missing. In this chapter, I investigated the impact of three different organic fertilizers on microbial communities associated with different hosts along the trophic chain. Grassland study sites in the field were fertilized with cow or horse manure, biogas digestate or pig slurry and control sites were only minimally managed by grazing flocks of sheep. Sample collection included samples of soil, phytosphere (i.e., plant-associated compartments: rhizosphere, endosphere and flowers of *Trifolium pratense*) as well as zoosphere (i.e., animal-associated compartments: feces of *Microtus arvalis, Arvicola amphibious*, the earthworm community and the gut of *Bombus lapidarius*). Using Illumina sequencing of the 16SrRNA gene we identified microbial profiles of these seven different compartments. Fertilization significantly increased the microbial diversity of soil and voles, while decreasing it in the rhizosphere and impacted the composition of all microbial communities along the trophic chain. Herein, below-ground compartments were affected more than aboveground compartments and pig slurry exerted the most significant effects. Different important genera overlapping between compartments imply certain ecological interactions that might induce cascading effects of changes in microbial communities along the trophic chain. Pig slurry-associated taxa were found in all trophic compartments, implying some direct effects of allochthonous taxa. However, low relative abundances indicated that these taxa did not fully establish. In contrast, soil nutrient analysis revealed that the composition of soil nutrients differed between fertilization regimes and explained over 50% of variation within the soil microbiome suggesting that changes in the microbial communities are rather due to strong indirect effects of differences in nutrient composition than an establishment of allochthonous communities. This chapter illustrates the impact of intensive fertilization, especially pig slurry fertilization, on different microbial communities along the trophic chain. The findings underline the importance of assessing different trophic compartments to get a full understanding of the effects of anthropogenic activities on ecosystems and their functions in line with the One Health frame work.

Chapter3: Effects of Pig Slurry Fertilization History on Grassland Microbiomes: Insights from Field and Mesocosm Experiments

Land use in grassland ecosystems usually reflects long-term management practices exerting legacy effects of fertilization on different microbial communities. As fertilizers are applied repeatedly, the introduction of allochthonous microbes and nutrients might lead to cumulative effects. However, also individual fertilization events can cause short-term changes in nutrient content and confront native microbial communities with foreign bacteria potentially resulting in shifts in microbial communities. This third chapter aims to compare the legacy effects of long-term fertilization history and short-term effects of intensive fertilization with pig slurry on the different microbial communities along the trophic chain. By establishing lab-scale indoor mesocosms consisting of soil, *Trifolium pratense* plants, earthworms (*Lumbricus terrestris*) and bumblebees (*Bombus terrestris*), I was able to recreate the grassland ecosystem in a controlled laboratory environment to simulate effects of repeated pig slurry application. Samples were collected for microbiome profiling before and after a fertilization event from the field site described in chapter two (reflecting long-term history of fertilization) and two mesocosm experiments (reflecting repeated fertilizer application) subjected to either pig slurry fertilization or no fertilization. Comparing the 16S rRNA gene sequencing from field and mesocosm samples revealed that soil and flowers of *Trifolium pratense* are solely impacted by long-term pig slurry application and legacy effects of long-term history of fertilization outcompete short-term effects. However, in root compartments, i.e., microbial communities of rhizosphere and endosphere, both long-term fertilization history and short-term pig slurry application impacted community composition and diversity. Such shifts in microbial community composition and a decline in diversity of the root reflect shifts in soil nutrient content. As a response to higher nutrient availability, roots immediately react to a single fertilization and reject microbes aiding in nutrient acquisition thereby decreasing diversity causing alterations in the composition. In contrast, genera with potential soil-remediating and pollutant-degrading properties were found to increase in abundance upon fertilizer application, thereby counteracting introduced changes potentially securing environmental health. Additionally, important genera overlapped between the field sites and the mesocosm set ups in root compartments. Such overlaps indicate that mesocosm experiments were successful in rebuilding the grassland ecosystem. This chapter shows, that the effects of a long-term history of fertilization as recorded from the field substantially impacts microbial communities along the trophic chain and outcompetes short-term effect of single applications. However, short-term application of pig slurry disturbs microbial communities in plant roots and cause an immediate reaction of the plant.

General discussion

Land use intensification is one of the major problems of the modern world threatening different ecosystems including grasslands. In this thesis, I investigated the impact of land use, specifically organic fertilizers and differences in their application history on different interconnected microbial communities along the grassland trophic chain. By including microbial communities along the soil-plant axis and interacting animals, I aimed to understand the full impact of fertilization on the ecosystem. After elaborating on the current status of knowledge on different abiotic and biotic stressors on soil microbiomes in chapter 1, the results in chapter 2 show that organic fertilization has host-specific effects on different microbial communities along the trophic chain, with pig slurry interfering most significantly with the microbial composition. Finally, to investigate the effects of fertilization history, I established indoor mesocosm experiments rebuilding the grassland ecosystem in a controlled laboratory environment. Comparing them to field sampling revealed strong legacy effects of long-term fertilization history on plant associated microbiomes as well as short-term effects of fertilization on root microbial communities.

Pig slurry fertilization exerts the most significant impact on microbial communities

Organic fertilizers, while more sustainable than synthetic ones in terms of plant-diversity and organic carbon content (De Vries et al., 2015; Shi et al., 2024; Villa-Galaviz et al., 2023), have previously been shown to tremendously affect microbial communities (Bebber & Richards, 2022; Gaube et al., 2020; Luo et al., 2015; Ma et al., 2021; Mora-Salguero et al., 2025; Romdhane et al., 2022; Schöps et al., 2018). The results of the field investigation (presented in chapter 2) and the experimental studies (presented in chapter 3) confirmed impacts of organic fertilizers, namely biogas digestate, cow and horse manure and pig slurry, on microbial communities across the trophic chain. Under long-term fertilization, microbial diversity in soil, rhizosphere and vole microbiomes as well as microbial community composition across all trophic compartments in grassland ecosystems, was altered, with pig slurry fertilization exerting most significant effects. These findings are consistent with previous studies reporting pig slurry exerting more significant impacts on differentially abundant genera in grass phyllosphere compared to poultry and bovine manure-treated plants (Tyrrell et al., 2023). The more pronounced effects of pig slurry relative to other organic fertilizers can likely be attributed to three main factors: differences in handling and storage (Tambone et al., 2010), variation in bacterial composition and the genetic potential of microbial members as well as the nutrient content (Tyrrell et al., 2023).

Storage and processing prior to application differ in the fertilizers applied to the grassland sites (described in chapter 2). While cows and horses disposed the manure directly onto the field during grazing, biogas digestate and pig slurry undergo storage or fermentation steps before application. Biogas digestates are usually the solid remains of an anaerobic digestion of animal manures, plant biomass, wastewater sludge or mixed substrates resulting in distinct microbial communities (Karimi et al., 2022). Anaerobic digestion of pig slurry in biogas plants reduces the load of certain pathogens and improves nutrient content and availability (Insam et al., 2015; Massé et al., 2011). In contrast, pure, undigested pig slurry is typically stored in farm tanks before field application allowing animal gut-associated bacteria to spread in the slurry. Consequently, stored but undigested pig slurry exerts the most significant effects on native microbial communities in comparison to biogas digestates and directly deposited cow and horse manure.

Manure introduces gut-associated microbes that have been suggested to carry antimicrobial resistance (AMR) genes (Heuer & Smalla, 2007; Y. Sun et al., 2021) altering the native microbial community composition. Indeed, the results of the source tracker analysis (presented in chapter 2), as well as the comparison of core microbiome to the composition of the slurry (shown in chapter 3), revealed the presence of pig slurry-associated genera across the different trophic compartments. However, the pig-slurry associated genera, i.e., *Clostridium sensu stricto 1, Terrisporobacter, Turicibacter*, and *Romboutsia* were only detected at low abundances and did not dominate the native microbial communities, suggesting that they are not the primary drivers of observed compositional changes. Nevertheless, fertilization might still cause an increase in AMR genes in the soil by activating the native soil microbiome and increasing the abundances of resident antibiotic-resistant bacteria (Semenov et al., 2021; Udikovic-Kolic et al., 2014). Indeed, repeated pig slurry application to grassland ecosystems has been reported to enhance diversity and abundance of AMR genes in soil and phyllosphere (Tyrrell et al., 2023; F. Wang et al., 2020). In contrast, aerobic composting or anaerobic digestion of manure or slurry reduces loads and disseminations of AMR genes in soil (L. Zhang et al., 2020), potentially explaining the distinct impacts of the three different organic fertilizers assessed in chapter 2.

Abiotic factors such as nitrogen and phosphorous availability are key drivers in shaping soil microbial communities (Fierer, 2017). Pig slurry applied to field sites (chapter 2 and 3) was associated with the highest levels of nitrogen input, likely explaining its greater impact on microbial communities compared to the other organic fertilizers. Soil nutrient analysis (presented in chapter 2 and 3) confirmed that pig slurry fertilized soils had the highest concentrations of macro and micro nutrients. Notably, over 50% of the variation in soil microbial composition could be explained by differences in macro and micronutrients, indicating a substantial direct effect of nutrient addition on the native

microbial communities. As outlined in chapter 1, elements such as nitrogen, organic carbon, iron, copper, zinc and manganese are key predictors of soil microbial community structure (Cederlund et al., 2014; Z. Peng et al., 2022; Sul et al., 2013). Due to varying nutrient requirements and functional potential among bacterial taxa, nutrient addition, specifically nitrogen and phosphorous, selectively promotes the growth of copiotroph, fast growing bacterial species (Leff et al., 2015) thereby directly altering the bacterial community composition.

Nutrient addition also indirectly affects soil microbial communities by altering soil pH, plant community composition and plant productivity (Dai et al., 2020; Leff et al., 2018). Soil pH has been shown to be one of the major drivers of soil microbial composition (Fierer, 2017) with long-term nitrogen input decreasing soil pH (Dai et al., 2020), which leads to less diverse microbial communities (Griffiths et al., 2011; Tan et al., 2020). Additionally, changes in soil nutrient content can shift plant community composition in grassland ecosystems (Avolio et al., 2014), often favoring fast-growing species, further straining soil microbial communities. Similarly, beyond plant community composition and species identity, plant developmental stage and associated growth also impact soil microbial communities (Fierer, 2017; Q. Peng et al., 2025). However, the results (presented in chapter 2) did not reveal differences in plant growth between sites fertilized with different organic fertilizers, suggesting that the observed shifts in soil microbial composition are rather explained by direct effects of fertilization induced shifts in nutrient composition as described above.

Belowground effects of fertilization are host-dependent

Microbial communities associated to different trophic compartments express host-specific profiles (Mallott & Amato, 2021) and land use has been shown to differently impact above and below ground compartments (Le Provost et al., 2021). Accordingly, the results of chapter 2 and 3 showed, that fertilization induced shifts in soil nutrients and comparably low abundances of pig slurry associated taxa, differentially affected microbiomes across the trophic compartments with significant effects on the below-ground communities. In particular, soil microbial communities mainly reflected increases in diversity after long-term fertilizer application, whereas microbial diversity in root compartments decreased already after a single fertilization.

As described in chapter 1, soil microbial communities are very sensitive to biotic and abiotic factors. Since soil is the compartment most exposed to addition of fertilizer, it is expected to be affect the most. Indeed, fertilizer addition has been shown to shift soil microbial composition after the initial application reflecting strong short-term effects (Kraut-Cohen et al., 2023; Lazcano et al., 2013). However, single pig slurry application in the mesocosms (as shown in chapter 3) did not alter soil microbial communities. This lack of immediate effects suggests that, in the short-term, native soil

microbial communities can outcompete pig slurry-associated taxa (Hamm et al., 2016) and nutrient-driven shifts are not expressed yet. Similarly, previous studies have shown that soil microbial communities can return to their initial composition after a single pig slurry fertilization (Suleiman et al., 2016), with no detectable manure-associated taxa observed 10 weeks after manure application (Tyrrell et al., 2023). These transient shifts reflect a certain short-term resilience of the soil microbial community to fertilization. In contrast, the data from long-term organic fertilizer application revealed increased soil microbial diversity and significant shifts in community composition. Such legacy effects of increased diversity and shifts in community structure due to long-term fertilization are consistent with previous studies (Kong et al., 2023; Luo et al., 2015; Y. Zhang et al., 2018) and likely driven by the slow release of nutrients and dissolved carbon during manure decomposition affecting the native microbial community (Dai et al., 2020; Leff et al., 2015; Mora-Salguero et al., 2025). Notably, such microbial shifts upon long-term application of fertilizers have been observed even years after suspension of fertilization (Y. Zhang et al., 2018) underlining the need for sustainable land use management practices.

Soil and rhizosphere are closely interconnected, with soil serving as microbial reservoir allowing plants to selectively recruit microbes into the rhizosphere (Turner et al., 2013). Thus, the soil microbiome is considered as the extended or second genome of the plant (Mendes et al., 2013). In line with that, the results of the source tracker analysis revealed overlapping genera between soil and rhizosphere, confirming this interconnectedness. Here, plants actively shape their rhizosphere by secreting sugars, organic acids, amino acids and phenolic compounds (Compant et al., 2019; C. X. Yang et al., 2025) to selectively recruit beneficial bacteria supporting them in drought stress mitigation, facilitation of nutrient cycling and uptake or pathogen resistance (C. X. Yang et al., 2025). At the same time, plants also exclude undesirable bacteria by secreting compounds with antimicrobial activity such as benzoxazinoids in cereal crops (Hu et al., 2018). These diverse root exudates make the rhizosphere a highly competitive habitat mainly sourced from the soil but exhibiting higher microbial activities and a changed microbial community in comparison to the bulk soil (Reinhold-Hurek et al., 2015).

The plant's selective pressure may result in different fertilization effects on rhizosphere communities compared to the bulk soil. Indeed, Shannon diversity of the rhizosphere microbiome decreased from minimally fertilized control sites to intensively fertilized pig slurry sites on the long-term. Additionally, as observed before (Schöps et al., 2018; C. Wu et al., 2023; Y. Yu et al., 2024) shifts in the community composition occurred upon long-term fertilization and additionally already after a single pig slurry application in the mesocosms. These changes in the community are attributed to immediate changes in plant root exudates in response to increased nutrient availability (Hao et al., 2022). Under fertilized conditions, plants are sufficiently supplied with nutrients and microbes that have been recruited in

nutrient poor conditions to help in nutrient cycling and uptake are no longer required, explaining the decline in rhizospheric alpha diversity. Consequently, nutrient-enriched soils have been shown to exhibit reduced rhizospheric nitrification as well as lower abundances of ammonia monooxygenase (*amoA*) genes (Xie et al., 2023). Fertilization and connected nutrient addition hence enhance environmental filtering of microbial communities (C. Wu et al., 2023). Interestingly, rhizosphere diversities in fertilized sites returned to the same level as in unfertilized sites after repeated fertilization in field and second mesocosm set up, but not after single application in the first mesocosm set up. This suggests that rhizosphere communities undergo transient changes in response to fertilization, with a repeated application causing cumulative effects (Meier et al., 2021).

Rhizosphere effects can further extend to the endosphere. Consistent with previous findings (Ma et al., 2021), I observed shifts in the endosphere microbial composition following long-term fertilizer application in the field, as well as after repeated fertilization in the mesocosms. However, in mesocosms, these changes were only observed after repeated pig slurry application, but not after the first single application. This indicated that short-term rhizosphere effects do not immediately translate into changes in the endosphere microbial composition. Given that the root colonization is mediated by the plant (Hodgson et al., 2025), such slightly delayed effect is expected and underscores the impact of fertilization history on the microbiome. The microbial transition from the rhizosphere into the root tissue involves overcoming the physical barrier of the roots as well as the plant immune system (Tzipilevich et al., 2021; K. Yu, Pieterse, et al., 2019). Microbe-associated molecular patterns (MAMPs) are released by bacteria and recognized by the plant immune system to activate immune responses (Newman et al., 2013). Importantly, the plant immune system responds to all bacteria, irrespective of their pathogenic or beneficial lifestyle (Newman et al., 2013; Teixeira et al., 2021; K. Yu, Liu, et al., 2019) to maintain microbial homeostasis and to prevent dysbiosis in plant associated microbiomes (Pfeilmeier et al., 2021). The fact that repeated fertilization still changes endophytic microbial communities reflects its great impact.

In line with observed changes in microbial diversities and compositions, important genera were differentially abundant between fertilized and unfertilized sites. The genera *Gaiella, Pseudonocardia, Sphingomonas* and *Nocardioides* were more abundant in soil and roots under long-term pig slurry fertilization. Except for *Nocardioides*, these genera were also identified as members of the core microbiome overlapping across different compartments. Prominent members of those genera can modulate endophytic microbial interactions as they are involved in the degradation of environmental contaminants, remediation of polluted soils, and in phytoremediation (Gatheru Waigi et al., 2017; S. Jiao et al., 2019; N. Li et al., 2021; Riahi et al., 2022; L. Wang et al., 2022). Further these bacteria have been found to change in abundance depending on the nitrogen levels in different soils (Meier et al.,

2021). Given that they represent important taxa in nutrient cycling, these taxa may shift from non-core to core members of the microbiome in response to environmental changes (Ye et al., 2022). Conversely, *Bacillus,* a genus known to harbor plant-beneficial bacteria, decreased in abundance upon fertilization as presented in chapter 2. These changes indicate a legacy effect of long-term pig slurry fertilization with the selective recruitment or rejection of specific genera may reflect an adaptive response of the plant to altered soil conditions, analogous to the cry for help theory. According to this theory, plants detect an attack by e.g., pathogens and activate internal defense responses which alter root exudates(Rolfe et al., 2019). These chemical changes recruit beneficial microbes into the root surrounding that inhibit pathogens or enhance nutrient uptake and immunity. Thus, plant actively modulate their environment to attract microbial allies(C. X. Yang et al., 2025). The results presented in chapter 2 and 3 suggest a similar mechanism, where, beneficial microbes are recruited to the rhizosphere to mitigate disturbances induced by fertilization.

Microbial communities of aboveground and belowground trophic compartments are interconnected

Effects of fertilization have primarily been shown for isolated compartments of the trophic chain mainly focusing on below ground soil or root associated communities (Meier et al., 2021; Tyrrell et al., 2023; Y. Yu et al., 2024). However, trophic interactions are key to ecosystem functions, thus, any changes observed in the soil and root compartments might cause cascading effects across connected trophic compartments (Gralka et al., 2020; Robinson et al., 2024; Van Der Putten et al., 2004). As outlined in chapter 1, soil forms the basis of terrestrial ecosystems and is in close interaction with different hosts and microbial communities located below-ground (roots, earthworms, voles) and above-ground (flowers, leaves and pollinators). Even though the verification of actual microbial transmission would require experiments using radioactively or fluorescently labeled microbes, which is not feasible in natural field set ups, the results of chapter 2 and 3 provided initial evidence for the different interactions of soil with other trophic compartments outlined in chapter 1.

Earthworms, belowground trophic members, displayed shifts in gut-associated microbial community composition upon long-term fertilizer application (see chapter 2 and 3), consistent with previous studies (Bi et al., 2021). Overlapping microbial genera between soil, roots, earthworms and voles identified as a part of source tracker analysis presented in chapter 2 as well as similar effects on microbial composition of roots and earthworms following long-term pig slurry application observed in chapter 3, suggested trophic interactions among these compartments. Comparable fertilization induced changes in microbial composition of roots and earthworms can be explained by the specific feeding habits of *Lumbricus terrestris.* As anecic earthworm species, they mainly feed on soil but also

on decaying plant material and plant roots (Doube et al., 1997; Valckx et al., 2011). Since earthworm gut microbial communities largely consist of what they ingest, observed shifts in rhizosphere and endosphere communities likely translate into changes in the gut microbial community of the earthworm (D. Liu et al., 2018). Given the key role of earthworms in nutrient cycling and pollutant detoxification in grassland ecosystems (Sun et al., 2020), changes in their gut microbiome could have significant implications for ecosystem functioning and health (Adomako et al., 2025). Similar transmission of diet-associated microbes has also been observed in herbivores (L. Zhu et al., 2021), partially explaining the microbial overlaps between soil, roots and voles observed in chapter 2. These findings suggest that fertilization-induced shifts in lower trophic compartments may translate into higher trophic compartments raising concern about cascading effects across the trophic chain.

In addition to belowground compartments, organic fertilization may also affect microbial communities associated to aboveground compartments such as flowers. Flowers form distinct microhabitats and have been shown to partially filter their associated microbial communities (Junker & Keller, 2015; Massoni et al., 2021). However, microbial composition of *Trifolium pratense* flowers was significantly altered by long-term fertilization as well as short-term fertilizer application in the field and first mesocosm indicating that flower associated microbial communities are still susceptible to external changes. In line with that, previous studies have shown that flower microbial communities homogenize due to intensive land use often characterized by a loss of rare taxa (Gaube et al., 2020). Such observed changes in flower microbiomes could be caused by direct fertilizer application to flowering plants. However, in the first mesocosm experiment, flower microbial communities differed between pig slurry fertilized and unfertilized set ups, despite the pig slurry being applied on mown plants that lacked floral tissues. Therefore, in the mesocosms, direct effects of pig slurry can be excluded and shifts must be introduced by other sources. Flowers are key compartments within the trophic chain and are in constant interaction with other plant and animal compartments. Microbial communities can hence be transmitted along the plant axis from roots, stems and leaves ultimately to flowers (Bai et al., 2015; Zhou et al., 2021). For instance, some *Streptomyces* strains have even been shown to travel along the vascular bundles to flowers (Kim et al., 2019). Thus, fertilizer induced changes in leaf microbial communities (Gaube et al., 2020) might cause cascading effects that alter the flower microbiome, explaining the effects seen in the first mesocosm set up. In addition, flowers may acquire parts of their microbial communities from the soil (Massoni et al., 2021). Evidence for such effects was observed in the results of the source tracker analysis which showed overlapping genera between soil and flowers. Overlaps may be caused by a variety of factors including e.g., air or splashes of water transporting soil-associated microbial communities to flowers (Butterworth & McCartney, 1991; Cevallos-Cevallos et al., 2012; Zhou et al., 2021). Additionally, ground nesting

pollinators, such as bumblebees, might not only import bumblebee specific microbes, but also aid to the introduction of soil associated microbes to the visiting flower (Keller et al., 2021; Kim et al., 2019; McFrederick et al., 2017). Indeed, the results of the source tracker analysis revealed overlapping genera between soil and bumblebees, supporting the potential introduction of soil-associated microbes. However, the same analysis did not show overlapping genera between flowers and bumblebees at fertilized field sites. Free-ranging bumblebees might prefer to forage on less fertilized field sites with a higher plant diversity and better resources (Birkenbach et al., 2024). As a result, they might have minimally interacted with flowers on highly fertilized field sites leading to a lack of overlapping genera. In the mesocosms set ups however, *Snodgrassella, Lactobacillus and Gilliamella*, known members of the bee gut core microbiome (Kwong et al., 2017; Z. J. Zhang et al., 2021), overlapped between flowers and bumblebees, suggesting an interaction between those two compartments. Within a natural ecosystem, such interaction might facilitate the transmission of microbes to pollinators (Keller et al., 2021; McFrederick et al., 2017; Russell et al., 2019; Vannette, 2020) enabling the dissemination of microbes between distant plants within the ecosystem.

Although, shifts in the microbial composition of a flower may not directly affect plant health to the same extend as root microbiome changes, alterations in the flower microbiome potentially impact plant reproductive success. Changes in the floral microbial composition can affect excreted sugars and amino acids, nectar volume and even the olfactory bouquet of a flower (Farré-Armengol et al., 2016; Schaeffer et al., 2019; Vannette & Fukami, 2018). In addition, pathogen presence can modify floral rewards by changing nectar composition (Vannette, 2020; Vannette & Fukami, 2018) hence changing resources for pollinators. These shifts in the chemical properties of a flower can make flowers less attractive to pollinators, potentially reducing pollination visits and hence negatively impacting reproductive outcome.

Mesocosms reproduce interactions of grassland ecosystems

I recreated the grassland ecosystem with repeated pig slurry application simulating a history of fertilization in grassland sites. Different overlapping microbial genera between field and mesocosms indicate that mesocosms could partially reproduce key ecological interactions. In the core microbial communities of rhizosphere and endosphere, *Rhizobium, Pseudomonas* and *Novosphingobium* overlapped between mesocosm and field samples irrespective of fertilization. These genera include important and well-known root-associated and plant-beneficial bacterial species (Chouhan et al., 2021; Y. Li et al., 2023; Molina et al., 2021; Santoyo et al., 2025). Especially *Rhizobium* species are able to fix nitrogen in legumes (Baxter et al., 2021) and different *Rhizobium* and *Pseudomonas* strains enhance biotic and abiotic stress resilience in plants (Sharma et al., 2008; Stringlis et al., 2018). Their

presence suggests that the lab-scale mesocosm set ups are successful in rebuilding the root environment and important associated ecological functions. Above-mentioned overlaps between flowers and bumblebee microbiomes in the first mesocosm set up further confirmed the simulation of key ecological interactions. Given that the second mesocosm set up reflected a history of repeated fertilization, the overlap of *Microvirga*, a flower specific genus (Ottesen et al., 2013), between the second mesocosm set up and field samples in fertilized samples implied, that mesocosms can at least partially recreate effects of long-term fertilization history. To fully simulate effects of all interactions and rebuilding almost natural conditions (Türke et al., 2017), more complex systems, like the iDiv ecotrons described in chapter 1, are necessary. However, overlapping microbial genera between mesocosm set ups and field samples in almost all compartments as well as similar effects of fertilization observed in field and mesocosms confirmed a successful recreation of grassland ecosystems in mesocosm set ups. As such, mesocosms aid in rebuilding key ecological interactions and are partially successful in simulating effects of a history of fertilization. Accordingly, mesocosms can help to understand the effects of fertilization on the leaf-flower-pollinator-axis in controlled conditions.

General conclusions and outlook

Amongst other abiotic and biotic factors, intensive land use, especially fertilization, impacts microbial communities associated to soil and plant compartments as well as interacting animals across the trophic chain of grassland ecosystem. With this thesis I present a broad examination of fertilization mediated land use effects on grassland microbiomes by combining a review on the current literature, a field study and controlled laboratory experiments recreating the ecological interactions. Summarizing the literature illustrated that soil microbial communities are mainly affected by factors like pH, soil moisture and temperature as well as the nutrient composition. In this respect, land use and fertilization play an important role in altering soil microbial functions like nutrient cycling, plant growth promotion or stress tolerance by changing soil nutrient content and introducing fertilizer-associated bacterial communities. The field study described in chapter 2 revealed that organic fertilization with biogas digestate, cow and horse manure as well as pig slurry alter microbial diversity, community compositions and the abundances of different microbial taxa with pig slurry causing most significant effects. The effects of fertilization however did not act in the same way across trophic compartments, but appeared highly host-specific. Accordingly, aboveground and belowground compartments were affected in different ways, with belowground compartments experiencing more significant shifts. These observed alterations in community composition in belowground compartments were mainly attributed to changes in soil nutrient content, while fertilizer introduced (pig-slurry associated) microbes remained low in abundance. Overlapping genera between different compartments suggested trophic interactions that potentially give room for cascading effects propagating fertilization-induced shifts in microbial composition across the trophic chain. The mesocosms described in chapter 3 successfully simulated the grassland ecosystem by recreating key ecological functions and observed trophic interactions. They partially confirmed field effects of fertilization on different microbial communities by simulating effects of a history of repeated fertilization. Overall, legacy effects of a long-term history of fertilization as observed in the field outcompeted short-term effects of individual pig slurry applications in the mesocosms. However, root microbial communities of *Trifolium pratense* experienced immediate effects of single pig slurry application, indicating an adaptive response by the plants. To fully recreate all long-term effects observed in the field, set ups need to be more complex and subjected to a longer history of recurring fertilization. Nevertheless, the mesocosm set ups were an effective measure to assess immediate effects and impacts of a short-term history of fertilization including key ecological interactions under controlled conditions. Taken together, the work compiled in this thesis shows that organic fertilization has nutrient-driven effects on different microbial communities along the trophic chain with

compartments exhibiting host-specific effects and that complex mesocosm set ups can recreate grassland ecosystems in controlled set ups.

This work follows the eco-holobiont framework (Singh et al., 2020) recognizing the importance of trophic interactions in shaping microbial communities across different trophic compartments. By assessing different plant and animal as well as soil associated microbial communities in the same set up, this thesis determines the effects of intensive organic fertilization on an ecological loop of interconnected microbial communities (H. Liu et al., 2019). Changes within one microbial community, e.g., the soil as the basis compartment in a terrestrial ecosystem, may cause cascading effects in the connected higher trophic compartments (Gralka et al., 2020; H. Liu et al., 2019; Robinson et al., 2024) propagating microbial shifts across the trophic chain. Additionally, leaf litter deposition (Veen et al., 2019), defecating animals or the impact of herbivores (Wolfgang et al., 2023) in turn alter soil microbiomes thereby closing the loop of microbial communities. This complex network of microbial interactions demonstrates that the observed effects on different microbial communities along the trophic chain may cause severe consequences for ecosystem health and functioning. In light of the One Health frame work, such global changes within the microbiomes of an ecosystem might not only affect plant and animal health but subsequently also translate to human health (van Bruggen et al., 2019). Thus, effective measures ensuring biodiversity in different compartments are necessary to secure sustainable agriculture, preserve food production and to ensure human well-being.

Given the significant effects of fertilization on microbial communities, reducing fertilization, would be a logical consequence to maintain microbial stability and ecosystem health. However, fertilization ensures agricultural productivity highlighting the need to establish compromises between agricultural productivity and ecological diversity in sustainable agricultural management practices. One potential compromise could be the application of smaller amounts or differentially processed organic fertilizers in combination with a stringent monitoring of pathogens and ARG abundances. Another approach, increasingly studied in agricultural research, is the use of plant growth-promoting bacteria to partially replace fertilizers and reduce the ecological impact. The artificial introduction of such plant beneficial microbes to the soil might support plant growth while reducing the need for intensive fertilization, ultimately providing a sustainable agricultural and improving fodder quality (Berg, 2009). However, the artificial introduction of microbes also alters the native microbial communities, potentially causing legacy effects. Therefore, such measures need to be taken with caution and accompanied by monitoring (Moore et al., 2022).

Taken together, the complexity of agricultural grassland ecosystems including plants, animals and their associated microbial communities as well as their interactions, require comprehensive

assessments of anthropogenic impacts such as intensive land use and fertilization to fully understand their consequences for environmental health. The disturbances in different microbial communities along the trophic chain outlined in this thesis, underscore the need for improved agricultural practices that ensure ecosystem health while simultaneously ensuring ecosystem productivity enabling a long-lasting sustainable agriculture.

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This dissertation is submitted as a cumulative dissertation and includes one published book chapter, and two published articles in peer-reviewed, international journals.

Advances in understanding soil microbiomes in ecosystem functioning across trophic chains

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Advances in understanding soil microbiomes in ecosystem functions across trophic chains

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1 Introduction

Soil is the basis for all agricultural development and one of, if not, the most important resources to feed humanity. While being the foundation of our well-being, soil microbiomes, defined as a consortium of all bacteria and fungi living in soil environments, host about 15% of global biomass (Bar-On et al., 2018). In addition to bringing in a huge biomass, soil is one of the most diverse biomes on our planet by hosting the most heterogeneous and diverse microbial communities (Xiong et al., 2021) and harboring around 25% of Earth's terrestrial species (Guerra et al., 2021). Soil microbiomes involve microbes with gene functions responsible for decomposition, climate regulation, plant growth promotion and stress tolerance, pest and disease control, degradation of pollutants and nutrient cycling and therefore display key players in ecosystem functioning (Hartmann and Six, 2023). The interaction between soil

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microbes and plants (i.e. plant–soil–feedback, the process of plants altering the biotic and abiotic soil properties which in turn alters the ability of plants to grow in this soil in the future) is one of the most important interactions in agroecosystems as it has the potential to support, e.g. plant growth and stress tolerance that are key to securing food supply for a growing world population, especially under changing climates. Considering plant microbiomes, soil-derived microorganisms are mainly associated with the rhizosphere and the roots themselves (Zarraonaindia et al., 2015). Microbial biomass and activity are up to 20 times higher in surroundings where roots and fungal hyphae are growing compared to bulk soil (Finzi et al., 2015; Kuzyakov and Blagodatskaya, 2015). The soil microbiome is therefore considered as a second genome that plants access and add to their functional diversity (Mendes et al., 2013).

In addition to being essential for the soil–plant interaction, soil microbial communities and their functions are, within the frame of the One Health concept, interconnected to all organisms living in the ecosystem and with that to ecosystem health. Understanding which factors affect the general soil microbiome functioning and what consequences a changing soil microbiome has for its essential services in ecosystem functioning is key to keeping a balanced One Health equilibrium with a healthy environment and healthy humanity (Banerjee and van der Heijden, 2023). In this review, we will discuss the effect of different abiotic and biotic factors on soil microbiome diversity and its functions from the perspective of complex ecosystems. This will include experimental setups to study ecosystem functions in soil–plant feedback in natural and controlled lab-scale environments.

2 Ecosystems in the One Health concept

An ecosystem describes the functional interrelatedness of all living organisms with the physical environment that they live in. Originally coined by the English botanist Sir Arthur Tansley in 1935, the term 'ecosystem' includes all elements, no matter whether they are chemical, physical, or biological, that can impact an organism under study (Golley, 1984). Since then ecosystems and their functions are studied intensively from all possible perspectives. By including not only animals, plants, and the physical environment like soil and water but also humans and by taking the assumption that health and well-being of humans are linked to the health of the ecosystem, the One Health concept emphasizes on the interaction and dependences of different partners in an ecosystem (Banerjee and van der Heijden, 2023). Microbes, although being the smallest partners in an ecosystem and not getting major attention in the context of One Health, play a pivotal role in ecosystem health. By interconnecting all components of the One Health concept and contributing to many ecosystem

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functions, the health of an ecosystem relies on the contribution of microbial communities (Berg et al., 2020).

3 Impact of abiotic factors on soil microbiomes

Soil is present in almost every ecosystem and subjected to several abiotic and biotic stresses that can change the composition of microbiomes and, eventually, its functions (reviewed in Fierer (2017)). Based on the physico-chemical properties of soils, it is difficult to generalize the impact of abiotic factors and climate change on soil microbial communities. While the impact of abiotic factors has been reviewed recently (Fierer, 2017; Jansson and Hofmockel, 2020), we will focus on some critical factors to explain how these changes in soil microbial communities can affect ecosystem functioning.

3.1 Impact of abiotic soil properties

Soil is not a homogeneous compartment but very diverse in its structure, pore size, permeability, mineral-to-organic matter composition, water content, and pH. The community composition of soil microbiota varies a lot in relation to these factors. A well-developed soil structure with a good drainage helps plant roots and fungi to properly establish and build a well-connected network, thereby allowing sufficient water and oxygen supply for microbes at different soil levels. This habitat structure raises microbial activities in the soil and supports nutrient cycling and supply to plants and other organisms (Fig. 3 in Hartmann & Six, 2023).

Several studies name soil pH to be the driving force determining soil microbial community composition (Fierer and Jackson, 2006; Lauber et al., 2009). About 43–85% of the variation in microbial community abundances involved in N-cycling at the landscape scale can be explained by environmental parameters, with pH being the most pressing one (Bru et al., 2011). Soils of low pH harbor more homogeneous communities that are dominated by *Acidobacteria*, whereas soils with a higher pH show a more diverse microbial population (Griffiths et al., 2011; Mukherjee et al., 2014; Tan et al., 2020). Bacterial diversity was found to be significantly higher in arable soils at pH 8 than at pH 4, whereas fungi were only slightly affected (Rousk et al., 2010). This might be explained by a higher availability of carbon and nitrogen substrate that, in turn, influences soil microbiomes (Kemmitt et al., 2006). Microbes rely on the availability of nutrients and hence the distribution of macro- and micronutrients plays a pivotal role in the community distribution of soil microbes. As main macronutrients, nitrogen availability (Cederlund et al., 2014) and soil carbon content (Sul et al., 2013) were found to predict soil microbial communities and

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distribution of microbes was further associated with several micronutrients, such as available iron, copper, zinc, and manganese (Peng et al., 2022).

3.2 Impact of climatic factors

Generally, a good supply of water in a well-structured soil promotes higher microbial activity, and soil moisture is one of the major factors influencing microbial diversity (Brockett et al., 2012; Frindte et al., 2019) with higher humidity of ecosystems (e.g. tropical rainforest) positively correlating with higher microbial biomass (Serna-Chavez et al., 2013). However, comparing different agricultural sites in China, Tan et al. (2020) found a negative correlation between mean annual precipitation and Shannon-index, describing (microbial) diversity within a sample based on species abundances and evenness (Shannon & Weaver, 1949). A fast increase in soil moisture, e.g. due to heavy rains or flooding on dry soil implies high-energy costs based on regulating osmotic stress to soil microbes and therefore might affect cell viability and integrity resulting in a release of mineralizable carbon (Halverson et al., 2000).

Temperature as another major climatic factor highly impacts the microbial composition in soil (Frindte et al., 2019). In an artificial soil warming experiment over 2 years, Nottingham et al. (2022) found a significant reduction of fungal and bacterial diversity causing large shifts in microbial community composition. In addition, a study on maize soils found higher temperatures to be negatively correlated with Shannon-index (Tan et al., 2020). In grasslands, experimental warming of soils for several years not only increased microbial biomass (Zhang et al., 2014) but also resulted in a shift in microbial community composition toward Gram-positive bacteria (Melillo et al., 2017) and a decreased fungal-to-bacterial ratio (Melillo et al., 2017; Zhang et al., 2014). Given that ecosystem functioning in soils is usually dependent on certain key taxa and complex interactions between components of the soil food web (Bardgett, 2002), this decline in soil microbial diversity can heavily impact ecosystem functioning.

By linking high temperature and low soil moisture, drought challenges soil microbial communities resulting in reduced microbial diversity (McHugh et al., 2017). With a lower water availability in the soil and therefore loss of connection between pores, resources are ill-distributed. Drought is therefore considered to reduce microbial functions that are important for ecosystems as previously reviewed in Schimel (2018) and Tecon and Or (2017). This might lead to reduced productivity under drought conditions or reduced carbon loss through suppressed respiration. However, it is difficult to make a general statement as soil properties, e.g. porosity and soil texture, strongly impact the accessibility of water and nutrients and therefore responses of soil microbial communities to drought (Patel et al., 2021). For instance, when looking at these factors in the long term, soil microbial communities might have the ability to

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adapt to slow changes in climatic conditions (Jansson and Hofmockel, 2018). A gradual change in climatic conditions might allow soil microbes to select heat- or stress-resistant genes or favor-respective species. A study on grassland and forest soils showed that historical exposure to changes in soil water content altered the ability of soil microbes to respond to new changes in soil moisture (Waldrop & Firestone, 2006) suggesting the existence of a microbiome-derived memory of ecosystems to operate under changing environmental conditions.

4 Impact of agricultural land use on soil microbiomes

Soils with a high biodiversity, especially high microbial diversity allow the different organisms to perform essential soil functions. A high biodiversity is therefore important to sustain human health (Wall et al., 2015). The cultivation of land for agricultural purposes has increased tremendously over the last decades in line with a growing global population and demand for food. The intensification of food production and agricultural land use has led to land conversion, soil perturbation, over-fertilization, intensive irrigation, and enhanced application of pesticides. These trends have led to soil degradation, environmental pollution, eutrophication, and greenhouse gas emissions (Matson et al., 1997). Intensive agriculture was further found to impact the resistance and resilience of the soil food web to drought (de Vries et al., 2012). To build up sustainable land use strategies and secure crop production, it is essential to understand the different responses of soil microbial communities to different stressors connected to such an intensive agricultural land use.

4.1 Impact of soil perturbation

Soil microbiomes are highly dependent on soil structure and soil properties (König et al., 2020) and any soil disturbance can have strong impacts on the functioning of soil microbiomes in soil organic matter (SOM) decomposition, carbon sequestration, and nutrient turnover. This can further feedback on soil–plant interactions (Six et al., 2006). The trend to larger farm sites requires larger and most importantly heavier machines and equipment for efficient tillage, sowing or harvest, leading to soil compaction in intensive agricultural land use. This compaction of soil structure can lead to a change in microbial community structure (Longepierre et al., 2021). It can further affect soil health and function and with that reduce productivity and ecosystem health and function (Hartmann and Six, 2023). Tillage exposes soils from deeper soil layers to the surface, increases macroaggregate turnover and by that disrupts the existing pore network, and increases SOM decomposition with a reduction in soil organic carbon stocks (Six et al., 2002). These changes in soil structure also heavily disrupt the hyphal network of fungi (Young and Ritz, 2000). Comparing

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different tillage intensities, soil organic carbon content in the upper soil layers was found to be higher in non-tillage as compared to conventional tillage soil (Hernanz et al., 2002). In addition, changes in soil density, macroaggregate structure, and pore connectivity can affect water drainage, nutrient availability, and oxygen penetration (Schäffer et al., 2008). As these factors are essential for microbial soil communities (discussed later), any changes in soil structure can harm microbial life in the soil.

4.2 Impact of fertilization

Soil nutrient content affects soil microbiota. In this respect, abiotic factors like soil moisture (as discussed above) in addition to mineral and organic fertilizers often composed of manure or slurry from cattle, pigs, or horses or digestates of biogas plants is especially relevant in agricultural fields. Several studies have shown short-term effects of different organic and mineral fertilizers on microbial biomass, community structure, and enzymatic activities (Guo et al., 2011; Lazcano et al., 2013). However, as fertilization in agricultural soils is usually not necessarily a short-term application but rather a long-term treatment, the focus shifts to understanding the effects of long-term application of fertilizers.

Long-term fertilization has a more persistent effect on microbial diversity and activity while organic farming increased microbial richness, decreased evenness, and shifted the structure of soil microbiota as compared to conventionally managed soils with mineral fertilization (Giacometti et al., 2014; Hartmann et al., 2015). Based on 107 datasets of 64 long-term experiments to determine the effect of mineral fertilizers, Geisseler and Scow (2014) showed that in most studies microbial biomass increased due to an increase in soil pH. In a 2-year field experiment, nitrogen application decreased the fungal/bacterial ratio whereas bacterial activity increased (de Vries et al., 2006) and long-term addition of nitrogen disrupts multipartite synergies between arbuscular mycorrhizal fungi and soil microbial communities. These synergies usually enhance plant and fungal nitrogen uptake from organic matter and a disruption leads to a reduced mycorrhizal nitrogen acquisition from organic matter and has therefore a tremendous impact on nutrient cycling and ecosystem functioning (Hestrin et al., 2019).

4.3 Impact of plants

Plants can benefit from soil microbes by receiving nutrients or other factors supporting plant growth and development. It is therefore not further surprising that plants shape the community composition of the soil microbiome thereby recruiting beneficial microbes from the soil that colonize the root endosphere (interior root part) or rhizosphere (immediate area surrounding roots). The

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root morphology and root exudates assist in this recruitment process (Rolfe et al., 2019; Saleem et al., 2018). In grassland, interactions between above- and belowground ecosystem compartments are so strong that plant species diversity could predict beta diversity of respective soil microbial communities (Prober et al., 2015). Certain mycorrhizal fungi, plant pathogens, or nitrogen-fixing bacteria (e.g. nodulating rhizobia) were found to associate preferentially with certain plant species and genotypes (Berg and Smalla, 2009; Hartmann et al., 2015; Lundberg et al., 2012). By changing several abiotic and biotic factors like oxygen pressure and carbon and nitrogen availability, plant roots also influence nitrogen conversion of soil microorganisms. Roots consume oxygen and thereby increase the anaerobic capacities of soils. In addition to a higher carbon availability (e.g. root exudates) in the rhizosphere, it promotes denitrification (Henry et al., 2008; Philippot et al., 2009, 2013). As microbial community structures differ between soil types, such rhizosphere effects can vary between soil type and plant species (Schreiter et al., 2014).

4.4 Impact of antibiotics and antimicrobial resistance genes

Antibiotics are one of the revolutionizing findings in modern medicine. Soil microbiomes are an important source of antibiotics. Certain habitats such as the nutrient-rich rhizosphere represent not only a microbiologically highly diverse but also competitive environment, where microbes produce and release antibiotics to control microbial competitors (Abrudan et al., 2015).

In some exceptional cases, antibiotics are directly applied to plants due to the lack of alternatives and the highly destructive nature of certain plant quarantine diseases (e.g. against fire blight in apple or pear orchards (Psallidas & Tsiantos, 2000)). By irrigation and drainage, these antibiotics can leach into the soil and reach the soil microbiota. Certain antibiotics such as sulfonamides can move into lower soil levels and ground water, but most antibiotic compounds remain in the upper layers of surface soil (Aust et al., 2010; Blackwell et al., 2009; Ostermann et al., 2013) where they can interact with other organisms interacting with the soil microbiome and soil compounds.

Livestock are often treated with antibiotics used in human therapeutics. Using manure and slurry for fertilization in the field can lead to an enrichment of antimicrobial resistance genes and bacteria in the soil (Heuer et al., 2011). Depending on the pharmacokinetics of antibiotics, antibiotic compounds can persist in the soil from months to years (Aust et al., 2010; Ostermann et al., 2013; Rosendahl et al., 2012). Repeated application of manure containing antimicrobial resistant agents as fertilizer might therefore lead to an accumulation of taxa containing possible pathogens and reducing other microbes contributing to a healthy soil microbiome (Ding et al., 2014). Manure is a potential source for antimicrobial resistance genes on mobile genetic

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elements (Binh et al., 2008; Wolters et al., 2014). Mobile elements such as broad-host range plasmids (Heuer et al., 2011) are a natural, evolution-based mechanism facilitating horizontal gene transfer (reviewed in Jechalke et al., 2014) that can equip soil bacteria, well adapted to the soil environment, with antimicrobial resistance genes. In fact, the transfer of antimicrobial resistance genes is enhanced by manure fertilization (Götz and Smalla, 1997; Smalla et al., 2000). This might lead to the persistence and spreading of resistance genes in the environment. An accumulation of resistance genes might sustainably affect the structure of soil microbiomes and consequently even translate in an altered overall ecosystem functioning.

5 Microbial communities in ecosystem functioning: plant–soil interaction

Soil microorganisms drive organic matter decomposition, humus formation, and nutrient transformation; processes most critical for crop productivity (Singh and Gupta, 2018; Van Der Heijden et al., 2008). Plants recruit their endosphere and rhizosphere microbiomes from the soil and rely on it for their development and reproduction even under changing environments. Prokaryotic fractions of rhizosphere communities are dominated by *Proteobacteria*, *Actinobacteria, Acteriodetes*, *Firmicutes*, and *Acidobacteria*, while in fungal phyla *Ascomycota* and *Basidisomycota* are the most common taxa (Mohanram and Kumar, 2019). Although arbuscular fungi (phylum Glomeromycota) and ectomycorrhizal fungi are well-studied, these groups represent only minor diversity among fungal communities from roots (Vandenkoornhuyse et al., 2002). Soil microbes can promote plant growth indirectly by fixing essential macro- (e.g. N, P, and K) and micronutrients (e.g. Fe) for the plant in the soil, either symbiotically in the endosphere of the roots, in a free-living lifestyle in the rhizosphere, or directly by producing plant growth promoting factors and plant hormones. Plant growth is further promoted by inducing stress resistance against abiotic stresses to the plant, e.g. the climate change factors drought, flooding, salinity, and heavy metal pollution as well as biotic plant stressors like herbivores (Mendes et al., 2013). To attract and interact with such beneficial microbes, plant roots excrete exudates such as sugars, organic acids, amino acids, and phenolics (Compant et al., 2019). This high nutrient environment makes the rhizosphere a quite competitive habitat and explains the distinct composition of microbial community in the rhizosphere as compared to the more diverse bulk soil community (García-Salamanca et al., 2013). Furthermore, above- and belowground plant microbiomes differ significantly, although they all share a certain amount of soil-derived microbes. It indicates, however, soil as the main source and reservoir for plant microbiomes (Edwards et al., 2015; Rochefort et al., 2021).

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Because of their significance in plant and ecosystem resilience under challenging environments, much attention was given to reveal how plant microbes impact plant traits related to nutrient acquisition, drought tolerance, disease resistance, and plant fitness (Philippot et al., 2013; Trivedi et al., 2020). Similarly, a focus was given on microbiome-based mechanisms that explain the outbreak of diseases. A dysbiosis, as an imbalance in microbiome structure, usually disturbs and reduces the biodiversity of microbial communities in the soil with potentially negative effects on plant fitness and ecosystem functions (Wagg et al., 2014). In the most extreme scenario, plant diseases occur as a consequence of a massive expansion of pathogens in a microbial community. Dysbiosis can be the result or the origin of a loss of soil biodiversity, which is in turn often accompanied by a reduction of plant diversity, productivity, nutrient retention, and belowground carbon allocation (Wagg et al., 2014). In the following sections, we will discuss these soil–plant microbiome interactions and their role in ecosystem functioning in the trophic chain and nutrient cycling.

5.1 Nutrient cycling

For all developmental processes from germination to reproduction, plants not only need sunlight, water, and carbon dioxide but are heterotrophs for macro- and micronutrients. For most vascular plants, these nutrients are taken up via roots from the soil. As discussed above, soil microorganisms are the main players in nutrient cycling and supply to different organisms associated with the soil. In addition, microbes produce hormones, e.g. used in quorum sensing but also with functions in plant growth promotion (Eichmann et al., 2021). Plant growth can be induced by the increase of nutrient availability or production of plant growth-promoting factors such as hormones that stimulate root growth or change the root system architecture for improved nutrient uptake.

5.1.1 Nitrogen

Nitrogen is one of the most limiting factors in plant growth and plants rely on the uptake of nitrogen from the soil environment as they cannot access atmospheric nitrogen. Nitrogen is usually present as nitrate, ammonium, and organic nitrogen in the soil, and soil associate bacteria can metabolize different forms of nitrogen (Näsholm et al., 2009). Plant roots, however, prefer inorganic nitrogen (i.e. nitrate and ammonium) rather than organic nitrogen which might affect the efficiency of nitrogen uptake (Bloom, 2015).

Nitrogen is available in the form of different nitrogen compounds that are the results of several reactions carried out by a complex network of microorganisms in the soil. These processes can roughly be separated into nitrogen fixation, nitrogen assimilation, ammonification, nitrification,

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denitrification, and anaerobic ammonia oxidation (Kuypers et al., 2018). Some of the intermediate products like ammonium resulting from nitrogen fixation and mineralization can directly be taken up by the plant or quickly processed to plant-available nitrate.

Arbuscular mycorrhizal fungi can acquire a substantial quantity of mineral nitrogen from organic and inorganic nitrogen sources and transfer it to the host plants (Hodge and Storer, 2014). Plants have therefore evolved mechanisms based on the release of the hormone strigolactone to attract mycorrhizal fungi and establish symbioses (Aliche et al., 2020; Kodama et al., 2022).

Acquiring nitrogen from organic or inorganic nitrogen sources is by far not restricted to arbuscular mycorrhizal fungi. Hestrin et al. (2019) showed that multipartite synergies between arbuscular mycorrhizal fungi and soil microbial communities enhance plant and fungal nitrogen acquisition from organic matter and microbial acquisition of plant photosynthates. Mycorrhizal fungi promote nitrogen acquisition but have limited ability to access organic nitrogen, while soil microbial communities mineralize organic nitrogen into bioavailable forms resulting in an up to ten-fold increase in nitrogen uptake.

Legumes further release flavonoids to attract N_2-fixing, nodulating rhizobia (Hassan and Mathesius, 2012). These symbioses can provide more than 300 kg of nitrogen per hectare per year (Bardgett and Van Der Putten, 2014; Fierer, 2017; Richardson et al., 2009).

5.1.2 Phosphorus

Phosphorus is another critical macronutrient for plants and other organisms. Although soils generally contain sufficient amounts of total phosphorus, these phosphorus resources occur predominantly as inorganic fractions that are adsorbed to soil mineral surfaces or organic matter (Richardson and Simpson, 2011). Only a small portion, namely orthophosphate anions (hydrogen phosphate (HPO_4^{2-}) and dihydrogen phosphate ($H_2PO_4^{1-}$)) are directly available for plants. Hence, plants usually depend on their microbial partners including mycorrhizal and endophytic fungi for phosphorus mobilization and supply (Bodenhausen et al., 2019; Jorquera et al., 2008). The underlying processes mediated by microorganisms can be grouped into three different mechanisms. First, they promote root growth and the resulting changes in root system architecture improve phosphorus uptake (Richardson and Simpson, 2011). Second, microbes can enhance phosphorus availability in the soil through mineralization and solubilization and, third, through assimilation and precipitation processes (Richardson et al., 2009). Arbuscular mycorrhizal fungi, for instance, are able to take up solubilized phosphorus from the environment and to translocate it directly to colonized plants subsequently (Kobae, 2019).

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In an extensive review of data from 399 papers published on a nationwide field survey in China, Li et al. (2021) found phosphate-solubilizing bacteria (PSB) and fungi to include *Bacillus*, *Pseudomonas*, *Enterobacter*, *Burkholderia*, *Penicillium*, and *Aspergillus*. Additionally, to these genera, a study on Argentinian acidic soil added bacteria from the genera *Pantoea*, *Acinetobacter*, and *Exiguobacterium* to the genera containing PSB. Some of these isolated strains showed high phosphorus-solubilizing activity in *in vitro* assays and promoted plant growth with higher P and N contents after inoculation (Collavino et al., 2010). A number of these fungi are endophytes with the ability to transfer phosphorus to their host plants to promote growth under low P conditions (Almario et al., 2017; Bakshi et al., 2015; Hiruma et al., 2016).

5.2 Plant growth-promoting factors – abiotic and biotic stress tolerance

Plant growth can not only be promoted by bacteria indirectly by transforming nutrients like, e.g. nitrogen or phosphorus into available forms for uptake by the plant but can also be promoted directly by the production of phytohormones and other signals. Plant fitness can further be enhanced by microbes promoting stress tolerance against abiotic stressors or by supporting plant defense against pathogens or herbivores (Trivedi et al., 2020). Different hormones are involved in plant–microbiome interactions and microbes and plants use the same hormone for different purposes. As hormones and their role in plant–microbiome interactions have been reviewed very detail recently in Eichmann et al. (2021), we will not elaborate on these interactions in detail, but we will give a short summary of how plant–microbiome interactions can promote plant growth by other mechanisms than nutrient cycling as described above.

Microbes can produce signaling compounds, i.e. plant hormones, which can enhance lateral root growth and branching and promote the development of root hairs for better nutrient acquisition (e.g. reaching microbiota-rich top soil levels) and water uptake from the soil. Nitrogen limitation induces primary root growth, enhances lateral root density and elongation, and root hair formation to help plants to overcome low phosphorus and nitrogen levels (Jia and von Wirén, 2020).

To cope with drought or floods, plants take advantage of their microbial partners and select a stress-resistance-promoting microbiome (Fitzpatrick et al., 2019; Naylor and Coleman-Derr, 2018; Xu et al., 2018). The plant hormone abscisic acid is involved in adaptation to abiotic stress, particularly in drought and salinity stress, and plays a pivotal role in modulating the plant immune signaling network (Asselbergh et al., 2008). It dampens the immune response and therefore facilitates shifts in the endophytic community composition (Fitzpatrick et al., 2018).

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Further, microbial activity can enhance plant fitness and survival by supporting the plant in its defense mechanisms against biotic stressors like pathogens or herbivores. As a first line of defense, plants recruit beneficial soil microorganisms against soil-borne pathogens by releasing plant root exudates (Mendes et al., 2011). This defense mechanism is based on the production of certain compounds like antifungal metabolites (Cha et al., 2016; Mendes et al., 2011) or volatiles (Carrión et al., 2018; Hol et al., 2015). Endophytic microbes can create a second layer of defense by enriching selected microbes that can produce enzymes and secondary metabolites against pathogens. Here, for instance, volatile compounds have been found to protect plants against insects (Hammerbacher et al., 2019; Sharifi et al., 2018). In addition, induced systemic resistance (ISR) can be promoted by the rhizosphere microbiome (Pieterse et al., 2014). As a result, plant infection can cause differences in root exudates that subsequently allow for establishing of further selected ISR-inducing bacteria. The promotion of jasmonic acid signaling by root-associated bacteria and fungi, e.g. also affects aboveground herbivory (Yuan et al., 2018).

Taken together, plants can defend themselves in several ways against several stressors by recruiting different beneficial microbes using root exudates and signaling compounds like phytohormones. Soil microorganisms play a pivotal role in this recruiting of beneficial microbes as they serve as a reservoir of microbes available to the plant (Edwards et al., 2015; Rochefort et al., 2021).

6 Soil microbiomes and ecosystem function

Ecosystems, as defined above, include all different organisms that interact with each other and their environment and all physical, chemical, and biological interrelations above- and belowground (Golley, 1984). Hence, in an ecosystem, there are not only plants that interact with soil microorganisms. Geophagy, so the consumption of soil, is very common in different animal species (Mahaney and Krishnamani, 2003) and soil microbes end up in animal guts with the potential to establish and contribute to the gut microbiome. By applying manure and slurry of livestock to the fields as fertilizer, gut microbiomes of farm animals again contribute to soil microbiomes. Accordingly, the eco-holobiont concept suggests a circular microbial loop which is formed by biotic (plants and animals) and environmental (e.g. soil, water, and air) microbiomes which can impact the assembly of microbiomes of all organisms within an ecosystem (Singh et al., 2020). To understand how changes in the composition and diversity of soil microbiomes affect the interacting partners within an ecosystem, in terms of ecosystem services, one needs to take several layers of an ecosystem and trophic layers into account.

Several studies have already shown effects on biodiversity and connected changes on ecosystem functions; however, they mostly focus on a few taxa

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and trophic networks. Assessing dynamics and effects of different biotic and abiotic factors on all these layers of an ecosystem is very complex and involves a lot of different compartments and species and, connected to that, different expertise. Therefore, cooperation platforms combining different research topics are needed. Cooperation platforms can contain a network for exchange of ideas and connect groups working on different topics and different sites like the Asia-Pacific Biodiversity Observation Network (Takeuchi et al., 2021). The Biodiversity Exploratories (www.biodiversity-exploratories.de, funded by the German Research Foundation, DFG) in Germany take the concept of a cooperation platform even further and support a joint effort to understand effects of land use on functional biodiversity on different levels of ecosystems and to assess the impact of biodiversity changes on ecosystem processes (Fischer et al., 2010). By combining around 40 different projects involving more than 250 researchers working on standardized forest and grassland plots subjected to different forest and agricultural land use practices, this research platform covers almost all aspects of biodiversity including not only individual species but whole groups of organisms from different trophic levels. Unraveling the impact of different environmental factors on soil microbiomes and their relations to other compartments of an ecosystem using such a joint research platform is highly needed to understand the effects of changing microbial diversity on ecosystem services (Fig. 1).

7 Systems to study ecosystem services across trophic chains in a controlled environment: mesocosms

Assessing ecosystem functions and effects of the natural environment on ecosystem compartments in the field are the first step to understand how different layers of an ecosystem interact and affect each other's ability to contribute to a healthy ecosystem and its services. In a second step, to study, for instance, the effects of future climatic conditions, land use intensification or diversity loss on ecosystem functions, controlled systems in a lab environment are a good tool, given that they can be manipulated to the interests of the study and the according ecosystem parts. Several studies use microcosm or mesocosm setups to assess different aspects of ecosystems in controlled environments to analyze the effect of individual factors on soil microbiome.

A broad range of factors, like the effects of several climatic factors (Rillig et al., 2019), the effect of invasive plant species (Stefanowicz et al., 2019), the effect of silver nanoparticles (Peixoto et al., 2020), or the effect of soil fauna diversity (Postma-Blaauw et al., 2006) on soil microbiome have been studied in such setups. By using large pots or tubes, most of these studies only include specific layers of an ecosystem and therefore focus only on specific interactions, e.g. soil–plant or soil–earthworm interactions. To include several

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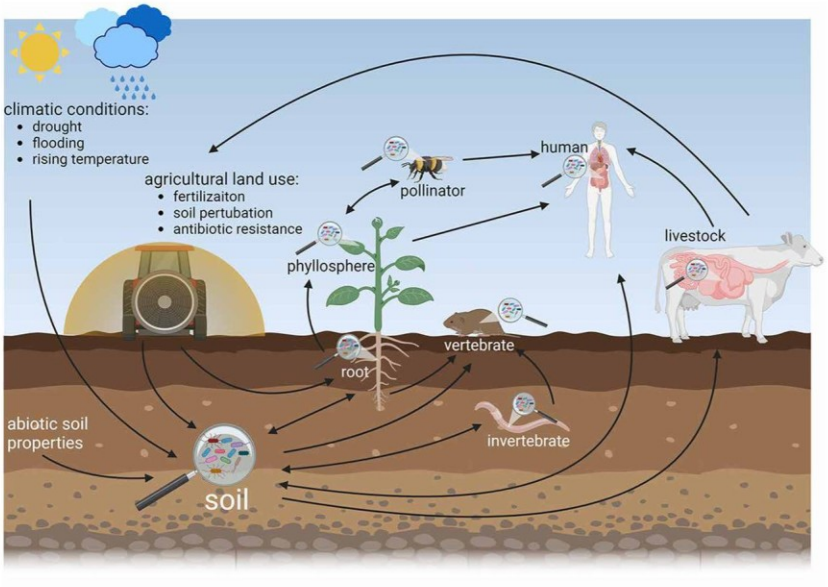

Figure 1 Biotic and abiotic factors affect soil microbial community composition and interrelations of soil microbiomes to other representative compartments within an ecosystem. The concept of the eco-holobiont suggests a circular microbial loop which is formed by biotic (plants and animals) and environmental (soil, water, air) microbiomes which can impact the assembly of microbiomes of all organisms within an ecosystem (Singh et al., 2020). Understanding how different factors alter soil microbial communities and their function is essential to unravel impacts on interrelations within the ecosystem and with that ecosystem services. Source: Figure created with BioRender.com.

layers of trophic interactions within an ecosystem, more complex setups are needed.

The iDiv Ecotron setup, led by the iDiv (German Center for integrative Biodiversity research) and Helmholtz Center for environmental research (UFZ) in Germany, builds an excellent research platform to study complex ecosystems and their functions under almost natural conditions by controlling for environmental conditions (Türke et al., 2017). This setup allows indoor multitrophic diversity research by manipulating above- and belowground trophic chains and studying the effects on diverse ecosystem functions, e.g. the effect of artificial light at night on soil communities and soil function (Cesarz et al., 2023) or the effects of aboveground predators on aboveground–belowground interactions and connected ecosystem functions (Schmidt et al., 2021).

The EcoUnits can be equipped with a broad range of ecosystem types, plant, animal, and bacterial species and a broad number of individuals thereby allowing biodiversity research from different biomes and assessing different facets of life. By controlling light, temperature, irrigation, fertilization, and

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species composition, the ecosystems can be manipulated to the specific factors affecting ecosystem services under study. For further information and visualization of the EcoUnits, please refer to Türke et al. (2017) and Schmidt et al. (2021).

Studies that focus on microbial biodiversity in different layers of an ecosystem, including plants, soil invertebrates, insects, and secondary consumers within a trophic chain of the ecosystem, are still lacking. Using a similar setup as the Ecotron would reveal a broader understanding of how microbiomes associated with different compartments of the trophic chain are regulated by a changing environment. Manipulation of different factors could help assessing changes and possible effects on ecosystem services.

8 Conclusion

Based on the One Health concept, which not only integrates animals, plants, and the physical environment but also humans, the interactions between different organisms and layers of the ecosystem are important. Accordingly, human well-being strongly correlates with the health of an ecosystem and its biotic and abiotic environment. Understanding the effects of different stressors on soil microbiomes and their functions within an ecosystem is necessary to maintain a sustainable and healthy environment. In this review, we discussed effects of different abiotic and biotic factors including those relevant in climate change and agricultural land use on soil microbiome diversity and its functions in complex ecosystems. In this respect, ecosystem functions of soil in plant growth promotion and stress tolerance reflects the interconnectedness of different organisms and the abiotic environment within an ecosystem.

To investigate ecosystem services and functions, different experimental setups need to be considered as provided in experimental systems such as the Biodiversity Exploratories, where different layers and various aspects of an ecosystem can be studied in a natural context. To reproduce and control such ecosystems and layers even more, we present the concept of indoor micro- or mesocosms, like the iDiv Ecotron, where different components and species of an ecosystem can be reconstructed under controlled conditions in the lab. These indoor ecosystems provide analytical platforms for further research on the role of microbiome diversity in the function of natural ecosystems and to identify the function of different ecosystem components in establishing a healthy and balanced One Health equilibrium.

9 Where to look for further information

Literature recommendations concerning the topics covered in this chapter are Fierer (2017) covering abiotic factors that drive soil microbial community composition, Hartmann and Six (2023) covering soil microbiomes in

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agroecosystems, Jechalke et al. (2014), who review the fate and effects of veterinary antibiotics in soil and Banerjee, and van der Heijden (2023), who describe soil microbiomes in the One Health concept. Concerning plant–soil interactions, Trivedi et al. (2020) describe several plant microbiome interactions and Eichmann et al. (2021) review plant hormones as communicators between plants and microbes.

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Chapter 2: Fertilization impacts microbiomes along the grassland trophic chain

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Fertilization impacts microbiomes along the grassland trophic chain

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Abstract

Agricultural grasslands are often managed intensively, influencing soil properties and microbial communities. These changes may, in turn, affect the microbiome of organisms across multiple trophic levels within the same habitat, and significant shifts in these communities can disrupt health and functionality along the entire trophic chain. This study investigates how fertilization affects microbial communities in multiple connected below - and aboveground trophic compartments of grassland ecosystems. We compared control grassland sites to those treated with organic fertilizers — biogas digestate, cow/horse manure, and pig slurry, using 16S rRNA amplicon sequencing and soil nutrient analysis. Shifts in microbial composition occurred in response to fertilization, with compartment-dependent effects. Changes were more pronounced in belowground compartments, with pig slurry fertilization exhibiting the most substantial impact. Overlapping bacterial genera detected among soil, roots, and higher trophic levels show the potential strong interactions across trophic levels shaping microbial communities. Pig slurry-derived microbial taxa were found in all compartments, but their low prevalence suggests an indirect effect of fertilization, primarily due to changes in nutrient availability. Compared to the control sites, pig slurry-fertilized sites showed proliferation of certain taxa, including *Clostridium, Ruminococcus* or *Lachnoclostridium*, particularly in the animal compartments. Our study highlights that the effects of fertilization permeate all trophic levels, with potential ecological and health implications aligned with the One Health framework.

Keyword: Fertilization, Organic fertilizer, Microbiome, 16S rRNA, Grassland, Land use, Trophic chain, Trophic interaction, One Health, Ecosystem monitoring

Introduction

On a global scale, intensive land use is a major driver of biodiversity loss and ecosystem dysfunction [1,2]. Apart from croplands, grasslands are the main component of agricultural landscapes in temperate regions, providing forage for grazers such as cattle and sheep. To increase the yield of hay and silage for livestock feeding, livestock slurry or manure are widely used fertilizers in grassland ecosystems. These organic fertilizers improve soil nutrient content and resource availability, thereby increasing plant productivity [3]. However, organic fertilizers not only affect soil properties directly through nutrients or pH changes, but also add livestock-associated microbial communities [4,5]. This microbial influx may displace natural members of the microbiota and introduce additional microbes with undesirable traits e.g. antibiotic resistance genes (ARGs) [6–8]. As microbial communities are known to overlap between organisms within the same habitat [9], such effects may extend to different trophic levels for e.g. from soil to higher trophic compartments- impacting both microbiomes and macrofauna, along with associated ecosystem functions. However, the extent of these impacts along the trophic chain remains largely undocumented [10,11].

Biogas digestate, cattle manure, and pig slurry are common organic fertilizers that vary both in their ability to promote plant growth and in their ecological impacts. Biogas digestate has a low carbon-to-nitrogen ratio that supports microbial and plant productivity without depleting soil organic carbon, contributing to agroecological sustainability [12]. However, it can retain contaminants from feedstock, including antibiotics, heavy metals, and pathogens [13]. Cattle manure, rich in organic carbon and nutrients, introduces intestinal microbiota that often fail to persist in soil but may include pathogens like *E. coli* O157: H7 and *Salmonella*, which can survive long after application [14]. Pig slurry, a nitrogen-rich fertilizer, is linked to high levels of ARGs and pathogens, largely due to intensive pig farming practices where animals are housed in confined conditions that increase infection risk [15,16]. Although pre-treatments such as composting, anaerobic digestion, and on-farm storage are commonly applied, the initial microbial composition of pig slurry remains a critical factor influencing its ecological impact [16]. On-farm storage may facilitate the survival and adaptation of gut-associated microbes, enabling their persistence in external environments, particularly in grassland soils. Unlike manure from grazing cattle or horses, which is deposited directly onto pastures, pig slurry thus may introduce a higher proportion of allochthonous microbes. Furthermore, the routine therapeutic use of antibiotics in intensive pig farming may contribute to the influx of antibiotic residues, abundant ARGs, and potential pathogens in the environment. Collectively, these factors suggest that pig slurry may exert the most pronounced influence on soil microbial communities and ecosystem processes.

Given the substantial microbial inputs and varying ecological footprints of these organic fertilizers, their application can significantly influence soil microbial communities and downstream ecosystem processes. There is growing evidence that intensification of land use and fertilization affects bacterial and fungal communities in soil, roots, as well as flowers and leaves, reducing the complexity of microbiome networks and causing microbial communities to become more homogeneous [17–20]. The diversity and stability of host-associated microbial communities, such as root-associated microbiomes in plants and gut microbiomes in animals, are crucial for host health [21–23]. These communities contribute to essential ecosystem services [7,21,24,25] or act as a barrier to the accumulation of antimicrobial resistances in the environment [26]. Disturbance of the microbiome might shift microbial composition and diversity, leading to dysbiosis characterized by a loss of commensals and a rise in pathogens, with potentially serious consequences for ecosystem function and host health. Therefore, based on the eco-holobiont framework and the One Health concept, it is important to move beyond examining specific compartments in isolation and instead consider how environmental perturbations like fertilization affect interconnected biotic and abiotic components across the trophic chain [7,11,27,28].

Although aboveground and belowground communities may be differentially affected by land use changes, they are ecologically interlinked through plant-mediated resource flows that define typical grassland trophic chains [29]. Soil microbes play a central role in this system by decomposing organic matter, recycling nutrients, promoting plant growth, and enhancing stress resilience. These interactions make soil–plant feedbacks essential for the functioning and stability of grassland ecosystems [30,31]. In turn, plants shape microbial communities by supplying resources and selectively recruiting beneficial microbes through rhizodeposition into the rhizosphere [23,32,33]. Various detritivorous and herbivorous animal species feed on belowground soil organic matter, roots and plant litter, as well as aboveground plant biomass, thus ingesting soil- and plant-associated microbes into their gut [34,35]. Similar to soil and rhizosphere, these (macro)organisms are also affected by fertilization-driven disturbances and may serve as reservoirs and vectors for zoonotic pathogens and antibiotic resistant bacteria [8,25,36]. Flowers can act as reservoirs for microbes [37]. As different pollinating insects share floral resources, flowers can function as transmission sites for microbes in pollinators, making microbe transfer between flowers and pollinators bidirectional [38,39]. These trophic interactions, and the importance of stable and balanced microbial communities for host and environmental health, reinforce the need to collectively study microbial communities associated with multiple links in the trophic chain to understand the impact of fertilizers on environmental health and ecological function. However, most studies investigating the effects of land use and fertilizers on microbial species richness and diversity have focused on individual trophic levels

in isolation, rather than considering the broader community of interacting above- and belowground trophic levels [1,40,41].

In this study, we investigated the effects of fertilization on microbial diversity, composition, and interconnectedness along the trophic chain — a linear network of interactions in a food web, beginning with producers and extending to consumers—within grassland ecosystems. In summer 2022, 494 samples were collected from seven trophic compartments across agricultural grasslands on the Swabian Alb (Germany), subjected to four fertilization regimes: control, cow and horse manure, biogas digestate, and pig slurry. Samples were categorized into the plant-related "phytosphere" and animal-related "zoosphere," with further distinction between aboveground and belowground compartments. Specifically, we aimed to investigate: (1) whether fertilization exerts similar effects on microbiomes across above- and belowground, as well as plant- and animal-associated compartments of the trophic chain, and (2) whether fertilization-driven microbial modulation shapes microbiomes across interconnected trophic compartments. We hypothesized that belowground communities with higher exposure to fertilization would be most affected, with pig slurry inducing the strongest impact by not only enriching soil nutrient reserves but also introducing elevated levels of ARGs and potential pathogens.

Our findings show that fertilization significantly affects belowground microbial diversity and composition across all trophic compartments, with pig slurry exerting the most pronounced influence. The limited abundance of pig slurry-derived taxa across trophic compartments, coupled with the substantial effect of soil nutrient content, suggests that these changes were primarily driven by altered soil nutrient levels rather than the establishment of slurry-derived taxa. The host-dependent nature of these responses highlights the importance of assessing fertilization impacts across trophic compartments within a One Health framework.

Materials and methods

Study area and sample collection

Sample collection was carried out between May and July 2022 on agricultural grassland sites located on the Swabian Alb, Germany (see Figure S1A) that were subjected to four different fertilization regimes typical for this region i.e. fertilization with (i) biogas digestate, (ii) cow/horse manure, (iii) pig slurry and (iv) control sites, i.e. former military sites that are minimally managed by grazing flocks of sheep (maximum twice per year). In total, 28 grassland sites were investigated, including seven control, eight biogas digestate, seven cow/horse manure and six pig slurry fertilized sites. Sampling

specimens were selected by targeting organisms that are representative of different trophic compartments above and belowground (Figure S1B) containing animals (zoosphere; i.e. feces of earthworms and voles (*Microtus arvalis, Arvicola amphibious*), bumblebee gut contents [*Bombus lapidarius*]) and plant-related samples (phytosphere; i.e. endosphere, rhizosphere and flowers of *Trifolium pratense*), as well as soil and samples from pig slurry. *T. pratense* L. is an important legume forage crop in temperate regions due to its high protein content [42] and the symbiosis with nitrogen fixing bacteria [43]. As *T. pratense* flowers are frequently visited by bumblebees and honeybees [44] and as several vole species prefer *T. pratense* roots as a food source [45–47], this system links above and belowground compartments in a widely distributed trophic chain in grassland ecosystems. In addition to microbial analyses, soil nutrient content and plant growth parameters (aboveground and root biomass, flower number, and plant height) were measured. Nitrogen input was estimated using the Biodiversity Exploratories land use index [48]. Detailed sampling and processing methods are provided in the supplement.

DNA extraction, library preparation and 16S rRNA gene amplicon sequencing

A total of 494 samples were collected (see Table S1 for sample sizes per compartment). To assess the bacterial composition, the V4 hypervariable region of the 16S rRNA gene was amplified in a two-step polymerase-chain reaction (PCR) using the primers 515 F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806 R (5'-GGACTACHVGGGTWTCTAAT-3') [49]. To avoid amplifying high amounts of chloroplast and mitochondrial DNA, peptide nucleic acid (PNA)-DNA clamps (mPNA and pPNA for plant (endosphere, flower) and mPNAs (bumblebee) samples) were added to the first PCR reaction [50]. Paired-end sequencing of the constructed libraries was conducted in two separate runs on an in-house Illumina MiSeq sequencing platform according to Fackelmann et al. [51] and Heni et al. [52] (see supplement for details). DNA extraction and sequencing of a total of 494 samples (excluding negative and positive control) resulted in 10,044,014 reads and 27,753 ASVs after filtering.

Soil nutrient assessment by electro-ultrafiltration

Soil samples were pooled per sampling site (n=23) for nutrient content analysis by the Bodengesundheitsdienst GmbH (https://www.bodengesundheitsdienst.de). Using electro-ultrafiltration (EUF), the concentrations of macronutrients (nitrate, organic nitrogen, phosphorus, potassium, sulfur, boron, magnesium, and calcium), micronutrients (iron, manganese, zinc, and copper), and sodium were determined for each site.

Bioinformatic sequence processing and statistical analyses

Sequence processing - removal of primers, denoising, removal of chimeras and merging of paired-end reads [53] - was conducted using the DADA2 pipeline in QIIME2 (version 2022.8) [54] (see supplementary material). All statistical analyses were performed in R and R Studio [55]. To evaluate the impact of fertilization on the microbiomes of all seven different trophic compartments, measures for microbial alpha diversity (Shannon index) and beta diversity (Bray-Curtis distances) were calculated for each compartment separately. The differences between the fertilization regimes within each compartment were evaluated using ANOVA with Tukey's HSD post-hoc test for alpha diversity and pairwise PERMANOVA (999 permutations) for beta diversity. The latter was conducted using the vegan package [56] and the pairwise Adonis package [57].

To determine how soil nutrients influence microbial composition, redundancy analysis (RDA) was performed using Bray-Curtis distances and available nutrients (P, K, S, N, NO_3, Na, Ca, Mg, B, Mn, Zn, Cu, Fe) as constraints. The ordiR2step function (vegan package) was used for forward selection to identify nutrients significantly explaining microbial variation. Using the ordinate function (microviz package) [58], we ran separate RDA models for macronutrients, micronutrients, and all nutrients to evaluate their impact on microbial community differences. Differences in nitrogen input and plant growth parameters across field sites were assessed using Kruskal-Wallis tests followed by pairwise Wilcoxon tests.

Differentially abundant taxa, i.e., taxa that respond to fertilization within the different trophic compartments, were identified using Analysis of Composition of Microbiomes (ANCOMBC2) [59,60]. First, samples from all three fertilization regimes were compared individually against those from the control sites. Samples from all fertilization regimes were then compared against each other (all pairwise comparisons). Core microbiome analyses were conducted for each trophic compartment individually, encompassing all pairwise comparisons between fertilization regimes to determine resilient taxa, i.e. taxa present in all samples within a compartment regardless of the fertilization regime, utilizing the core function in the microbiome package (detection cut-off: 0.0001, prevalence cut-off: 0.5) [61].

Subsequent analyses were conducted on a refined dataset, comprising only those ASVs identified as responding taxa in the differential abundance analysis and as resilient taxa in the core microbiome analysis. To verify the impacts of fertilizer application on microbial composition within the various trophic compartments, the refined dataset was employed to conduct a canonical analysis of principal coordinates (CAP) [62], followed by PERMANOVA and pairwise PERMANOVA. Based on interactions among trophic compartments, a Source Tracker2 [63] analysis was conducted to assess microbial taxa

that overlap between compartments and to elucidate potential exchanges of taxa between compartments. All graphs were created in R using ggplot2 [64].

Results

Impact of fertilization on microbial alpha and beta diversity in different compartments along the trophic chain

Microbial alpha (ANOVA: Shannon, $p<0.001$) and beta diversity (PERMANOVA: Bray-Curtis, df=3; R2=0.027, p=0.001) were highly variable between trophic compartments and fertilization regimes (Figure 1). Disentangling the overall significant effect of fertilizer application on alpha diversity revealed that Shannon diversity was significantly impacted by fertilization in the belowground compartments- soil (ANOVA: $p=0.029$) and rhizosphere (ANOVA: $p<0.001$) as well as in the belowground zoosphere compartment voles (ANOVA: $p=0.001$) (Figure 1A, Table S2). In soil samples, alpha diversity was increased in pig slurry fertilized sites when compared to biogas but not to control or cow/horse manure fertilized sites (TukeyHSD: biogas vs. pig: $p=0.029$) (Table S2). For voles, alpha diversity was increased in biogas- and cow/horse-manure-fertilized sites (TukeyHSD: control vs. biogas: $p<0.001$; control vs. cow/horse manure: $p=0.003$) (Table S2). Moreover, alpha diversity of the rhizosphere was significantly lower in pig slurry-fertilized sites as compared to all other treatment groups (TukeyHSD: control vs. pig: $p<0.001$; biogas vs. pig: $p<0.001$, cow/horse manure vs. pig: $p=0.049$; biogas vs. cow/horse manure: $p=0.026$) (Table S2). Flowers and bumblebees, representing the aboveground trophic compartments, as well as earthworms exhibited no differences in alpha diversity (Figure 1A). Similarly, the root endosphere showed no significant variation under the different fertilization regimes (Table S2). Hence, the impact of fertilizer application is more pronounced in belowground compartments.

Beta diversity was significantly affected by fertilization regimes in all trophic compartments (Figure 1B). Within the phytosphere and soil compartments, we noted significant differences in beta diversity revealing an impact of all four fertilization regimes on microbiome compositions (soil: $p=0.001$, rhizosphere: $p=0.001$, endosphere: $p=0.001$, flower: $p=0.001$; PERMANOVA: Bray-Curtis) (Table S3). Similarly, differences in beta diversity between fertilization regimes were observed in all zoosphere compartments (voles: $p=0.001$; earthworms: $p=0.005$; bumblebees: $p=0.001$ PERMANOVA: Bray-Curtis) (Table S3). In voles, microbial composition differed between all fertilization regimes except for comparisons between control and pig slurry fertilized sites (pairwise PERMANOVA: $p=0.656$) (Table S3). Pig slurry and cow/horse manure fertilization altered earthworm microbiomes (pairwise

PERMANOVA: control vs. pig: p=0.021; control vs. cow: p=0.002) (Table S3) and fertilization with biogas digestate impacted the microbial compositions in bumblebees (pairwise PERMANOVA: control vs. biogas: p=0.023; cow vs. biogas: p=0.001; pig vs. biogas: p=0.005) (Table S3). Overall, these analyses revealed an impact of fertilization on microbial communities of all trophic compartments and pig slurry appeared to have the most pronounced effects.

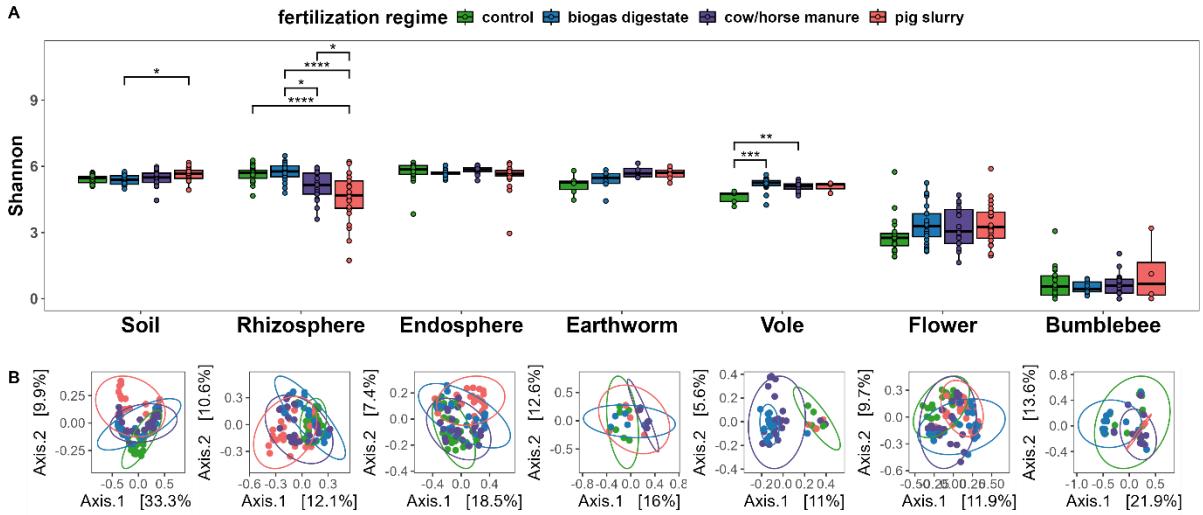

Figure 1: Effects of fertilization on microbial diversity. (A) Alpha diversity (Shannon index) and (B) Beta diversity (Bray Curtis) measures for individual compartments along the trophic chain. ANOVA followed by post hoc Tukey's HSD test was used to analyze effects of fertilization regimes on alpha diversity (*=p=0.05, ** p<0.01, *** p<0.001 **** p<0.0001). Differences in beta diversity were assessed using PERMANOVA followed by pairwise PERMANOVA.

Effects of soil nutrients on soil microbial communities and plant growth

Redundancy analysis (RDA) was used to model the amount of variation between soil microbial communities of different samples based on Bray-Curtis distances that can be explained by the different soil nutrients. Soil electro-ultrafiltration provided the content of eight macronutrients (P, K, S, N, nitrate, Ca, Mg, B) and four micronutrients (Mn, Zn, Cu, Fe) as well as the sodium content in soil samples. Forward selection of significant values revealed non-significant impacts of zinc and magnesium. After selecting significantly impacting nutrients, the model explained 51.9% of variation between the different soil microbial communities. Selected macronutrients explained 38.9% of variation (adjusted R2=0.389) (Figure 2A) while selected micronutrients including sodium explained 28.2% of variation (adjusted R2=0.282) (Figure 2B) between microbial communities of different samples. Out of all tested nutrients, calcium explained the most variation (19.2%), followed by phosphorous (11.1%) and nitrate (9.1%) while only phosphorous contents were significantly different between soils collected on the four different fertilization regimes (ANOVA: p=0.035) (Figure 2C).

Nitrogen input (kg[N]) per field site was calculated based on landowner information (Figure S2A). Inputs varied significantly across fertilization regimes (Kruskal-Wallis, $p < 0.001$), with all pairwise comparisons significant: control sites had the lowest input, followed by cow/horse manure, while biogas and pig slurry sites received the highest nitrogen levels (Table S4). To assess the impact on plant growth, we measured aboveground dry biomass, root fresh biomass, plant height, and flower number in *T. pratense* (Figure S2B). Aboveground biomass ($p = 0.200$) and flower number ($p = 0.610$) did not differ significantly between treatments. However, root biomass differed between control and cow/horse manure sites ($p = 0.046$), and plant height showed overall differences ($p < 0.001$), being significantly greater on biogas and pig slurry sites compared to control and cow/horse manure sites.

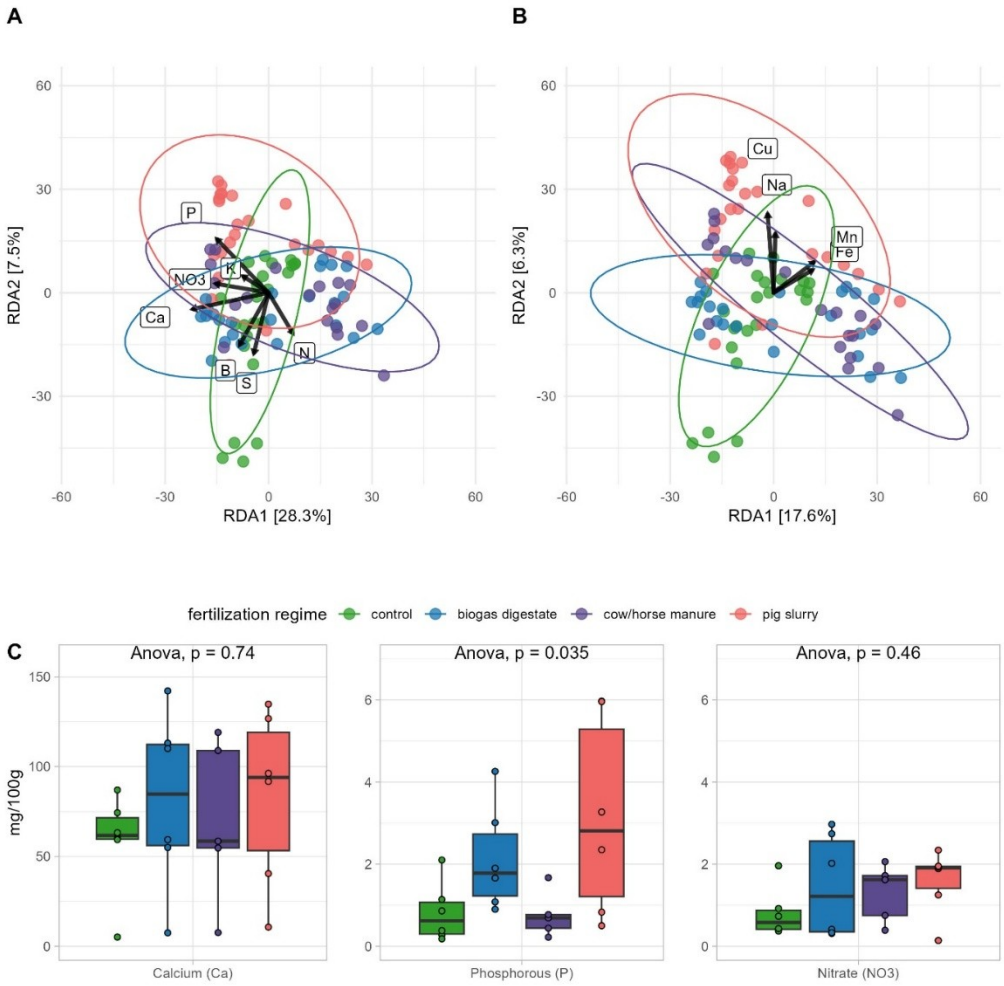

Figure 2: Soil nutrient content analysis. Redundancy analysis (RDA) showing the effects of (A) macronutrients and (B) micronutrients on Bray-Curtis distances of soil microbial communities. Arrows indicate the direction and identity of soil nutrients associated with microbial community variation. Colors represent fertilization regimes. (C) Soil concentrations of the three key nutrients (Ca, P, and NO_3^-) driving microbial community differences are shown, with ANOVA assessing differences between fertilization treatments.

Fertilization impacts on resilient and responding bacterial taxa along trophic compartments

We assessed the impact of fertilization on microbial communities across trophic compartments using ANCOM-BC2, identifying 600 differentially abundant ASVs from 82 genera. These were classified as *responder taxa*—taxa whose abundances significantly changed in response to fertilization, indicating sensitivity to nutrient input (Figure 3A, Figure S3). To identify taxa persisting across treatments, we performed a core microbiome analysis (prevalence ≥ 0.5, detection ≥ 0.0001) within each compartment, identifying 894 ASVs from 159 genera classified as *resilient taxa* (Figure S4). Comparison revealed 57 overlapping genera, termed *fertilization-responsive core genera*—taxa consistently present yet responsive to fertilization, suggesting ecological adaptability (Figure 3B).

In the phytosphere (excluding flowers), soil, and earthworms, *resilient* genera were more abundant than *responders*, and all detected *responder* genera were subsets of the *resilient* group (Table S5). This suggests that fertilizer-induced compositional changes in these compartments were primarily driven by changes in the abundance of *resilient-taxa*. In contrast, bumblebees and voles harbored fewer *resilient* than *responder* genera, with limited overlap between the two groups, indicating that microbial shifts in these compartments were likely driven by opportunistic, non-core taxa (Table S5).

Seven *responder* genera were shared across at least two trophic compartments (Figure 3A). Among these, *Bacillus, Luedemannella*, and *Streptomyces* consistently decreased in abundance at fertilized sites in soil, roots, and earthworms. In contrast, *Gaiella, Massilia*, and *Nocardioides* were enriched at fertilized sites in soil, endosphere, flowers, and bumblebees (Figure 3A). Among *resilient-taxa*, five genera were core to multiple compartments. Notably, *Bacillus, Microvirga, Pseudonocardia*, and *Gaiella* were prevalent in all belowground and plant-associated compartments (soil, rhizosphere, endosphere, earthworm gut, and flower) (Figure S4). *Sphingomonas* was also core to the rhizosphere, endosphere, earthworm gut, flower, and bumblebee microbiomes. Voles exhibited no overlapping core genera, indicating a highly host-specific microbiome (Figure S4).

Importantly, *Bacillus* and *Gaiella* emerged as key fertilization-responsive core genera- both core to multiple compartments and differentially abundant. Bacillus declined with fertilization, suggesting that it is negatively affected by nutrient enrichment, while *Gaiella* increased, indicating a potential advantage under fertilized conditions.

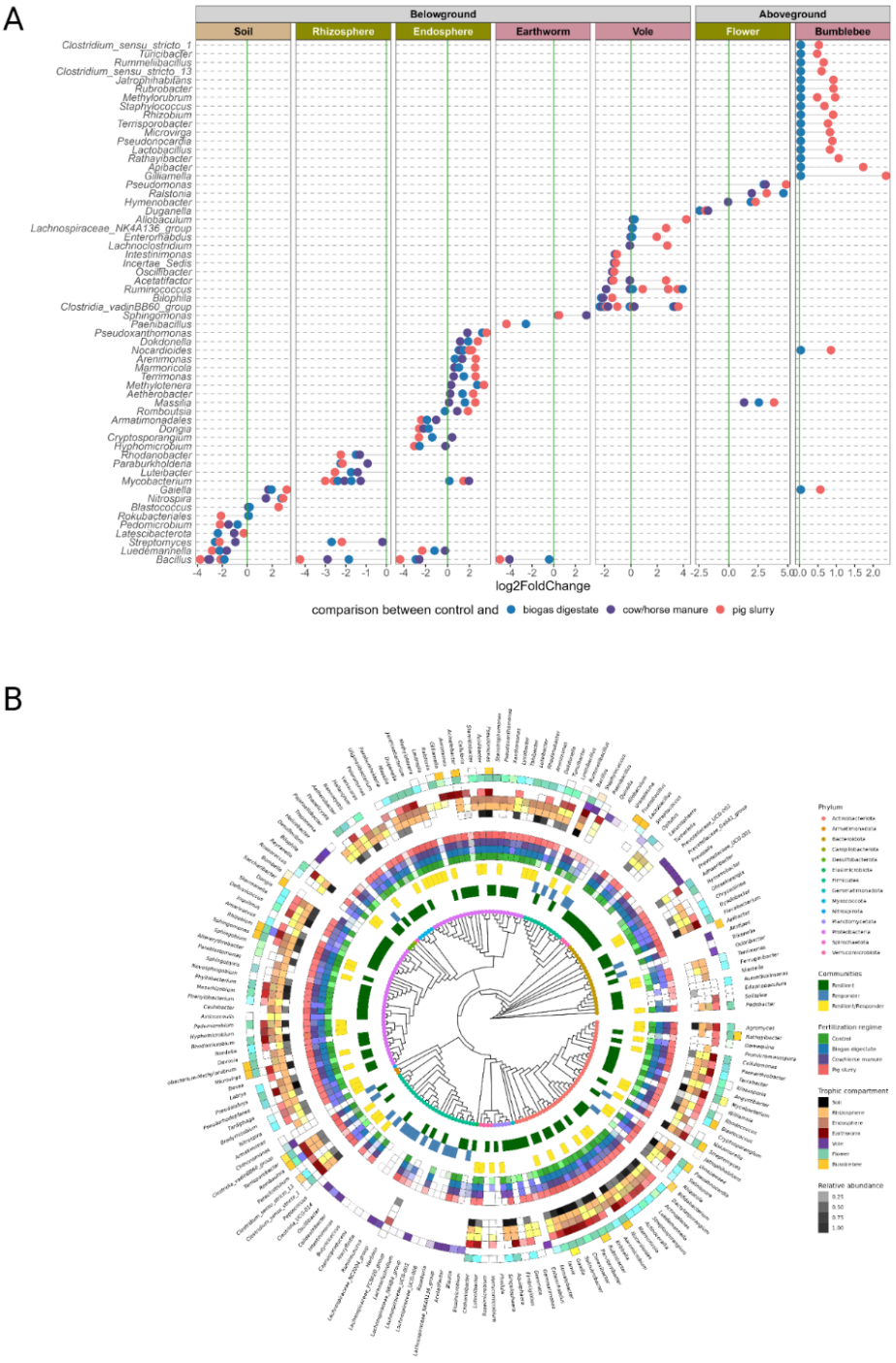

Figure 3: Bacterial genera and taxa responding to fertilization within the different trophic compartments. (A) Differentially abundant genera between unfertilized control sites (vertical line) and sites fertilized with biogas digestate, cow/horse manure and pig slurry, respectively, were identified using ANCOMBC2. Phytosphere compartments, zoosphere compartments and soil are marked with different colors in the title bar. Positive values of log2fold change represent genera that are more abundant on fertilized sites; negative values represent genera that are less abundant on fertilized sites compared to control sites. (B) Responding (differentially abundant) and resilient taxa (members of the core microbiome) according to fertilization regimes and trophic compartments they were found in.

Identification of overlapping taxa between different trophic compartments

To examine microbial communities overlapping across trophic compartments, we performed SourceTracker2 analysis on a refined dataset containing both *resilient* and *responder-taxa* (Figure 4). Potential microbial sources - including soil, rhizosphere, endosphere and flowers - were defined based on their positions in the trophic chain. All analyzed interactions are summarized in Table S6.

Within the phytosphere (using soil, rhizosphere and endosphere as source), overlaps were observed between soil and rhizosphere (57 genera), rhizosphere and endosphere (47 genera), and soil and flowers (53 genera). In earthworms, substantial microbial overlap was detected with soil (53 genera), rhizosphere (42 genera), and endosphere (32 genera). In contrast, voles exhibited minimal overlap, sharing only two genera with soil, rhizosphere, and endosphere, while bumblebees overlapped in one genus with soil and did not have any overlapping genera with flowers (Figure 4).

While taxa classified as *resilient* only showed few overlaps across compartments (Figure 4A), most overlapping genera identified by SourceTracker2 corresponded to *fertilization-responsive-core* genera (i.e., both resilient and responder taxa, Figure 4B). Prominent *resilient* genera were overlapping across multiple compartments (excluding voles and bumblebees) included *Gaiella, Mycobacterium, Solirubrobacter, Nocardioides, Pseudomonas, Kineosporia, Rubrobacter, Mesorhizobium, Parviterribacter*, and *Phyllobacterium* (Figure 4A). Likewise, *Pseudonocardia, Bacillus, Conexibacter, Pseudolabrys, Streptomyces, Agromyces, Microvirga, Pedomicrobium, Kribbella*, and *Acidibacter* emerged as the most prevalent *fertilization-responsive-core* genera spanning multiple compartments (Figure 4B). Notably, *Bacillus* and *Romboutsia* were present in all belowground compartments, including voles. Selective overlap of *Rhizobium* and *Paenarthrobacter* was observed between soil, rhizosphere, and endosphere.

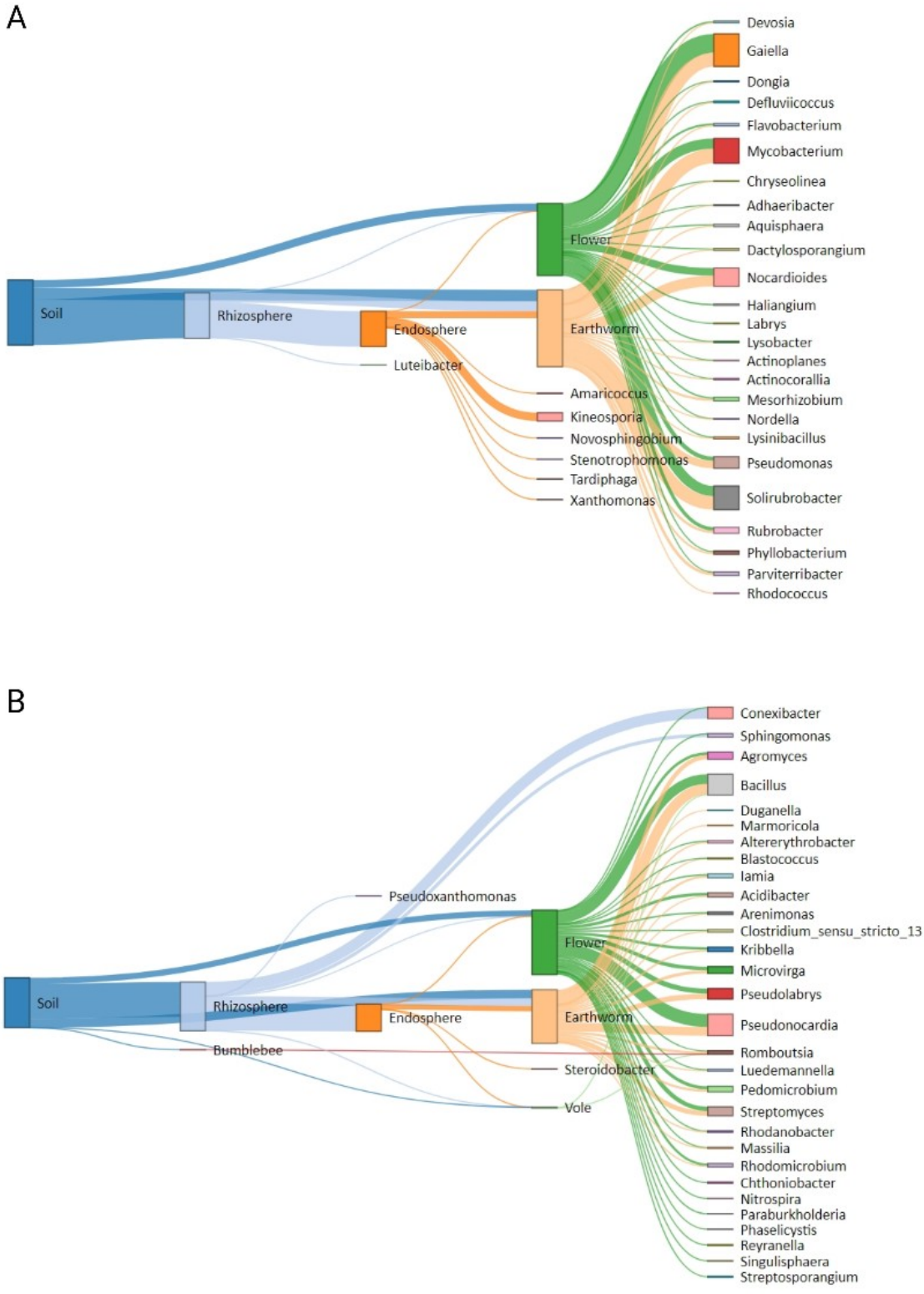

Figure 4: Proportions of bacterial genera overlapping between source and different target compartments along the trophic chain analyzed by SourceTracker2 analysis. (A) Results based on genera identified as resilient taxa, (B) Results based on genera identified as both, resilient and responding taxa. In both analyses, the source compartments were soil, rhizosphere and endosphere while key target compartments were endosphere, flowers, earthworms and voles.

To test the hypothesis that pig slurry may introduce allochthonous communities, we analyzed the microbial composition of pig slurry and its overlap with all trophic compartments. We noted *Clostridium sensu stricto* 1, *Terrisporobacter, Pseudomonas, Turicibacter, Lactobacillus*, and *Romboutsia* as the dominant genera in pig slurry (Figure S5). These genera were detected across the trophic chain at relative abundances of 1–5%. Four genera viz. *Clostridium sensu stricto* 1, *Terrisporobacter, Turicibacter*, and *Romboutsia* were overlapping between pig slurry and other compartments (Figure S6). Among them, *Turicibacter* was detected in all compartments including bumblebees, while *Terrisporobacter* and *Romboutsia* were found in flowers and all belowground compartments, excluding voles.

Despite overlaps in microbial genera across compartments and treatments, constrained analysis of principal coordinates (CAP) revealed clear separation of microbial communities by trophic compartment (PERMANOVA: $p = 0.001$, $df = 6$, $R^2 = 0.389$) and by fertilization regime across all compartments (PERMANOVA: $p = 0.001$, $df = 3$, $R^2 = 0.030$) (Figure S7). Pairwise PERMANOVA showed that each trophic compartment hosted a distinct microbial community, with all comparisons significantly different (Table S7). Likewise, fertilization regimes differed significantly from one another regardless of compartment (Table S8). These results indicate that microbial communities were consistently shaped by both compartment and fertilization regime.

Discussion

Intensified land use can have significant effects on grassland biodiversity, spanning from microbes to fauna. We investigated whether manure-based fertilizers influence microbiomes across the grassland trophic chain. Our findings revealed that pig slurry, in particular, induced marked, host-dependent shifts. The study demonstrates that fertilization alters microbial diversity and composition across the trophic chain, with the most pronounced effects observed in belowground compartments. The strongest shifts were noted in soil, rhizosphere, and voles, suggesting heightened susceptibility in these compartments. While microbial composition remained largely host-dependent, we identified a core set of fertilization-responsive genera across compartments, highlighting the interdependence of trophic levels and underscoring the need for a comprehensive evaluation of fertilization impacts.

Impact of Fertilization on Microbial Diversity Across Trophic Compartments

Microbiome analyses revealed contrasting effects of fertilization on microbial alpha diversity: it increased in soil and voles, but declined in the rhizosphere. These opposing trends likely reflect

differences in ecological and physiological dynamics. Fertilization introduces both nutrients and allochthonous microbes. While the rhizosphere is shaped by host-driven selective pressures, soil is more permissive, allowing introduced microbes to establish more easily and potentially boosting diversity under intensive fertilization. Although soil and rhizosphere are closely linked, we observed a gradual but significant decline in microbial diversity in the rhizosphere, from minimally fertilized control to pig slurry-fertilized sites. This decline was associated with higher nutrient availability and a reduction in plant-beneficial bacteria involved in nutrient cycling under intensive land use [3,65–67]. Plants actively shape their root microbiomes through rhizo-deposition, selectively recruiting or excluding microbes [68,69]. Indeed, fertilized sites in our study exhibited elevated phosphorus and nitrate (NO_3) levels, with the highest concentrations found on pig slurry-treated sites. Consistent with these findings, reduced nitrification and lower *amoA* gene (ammonia monooxygenase) abundance were observed in nutrient-enriched soils, particularly those treated with pig slurry and biochar [70]. These results suggest that nutrient enrichment indirectly influences microbial community assembly in the rhizosphere. Expanding on this, we observed contrasting effects of fertilization on above- and belowground trophic compartments, with alpha diversity most significantly impacted belowground, especially under pig slurry application. Similarly, Le Provost et al. [1] found divergent responses between above- and belowground compartments across a land-use gradient, including fertilization, in their analysis of over 4,000 taxa from grassland ecosystems.

Fertilization-Induced Shifts in Microbial Composition and Soil Properties

Fertilization also altered microbial community composition, with assemblages across all compartments clustering according to the fertilization regime. However, the strength of this effect varied by trophic compartment. In soil, macronutrients and micronutrients together accounted for over 50% of the variation in microbial community structure, with calcium, phosphorus, and nitrate being the most influential factors. These results suggest that fertilization shapes soil microbiomes primarily through indirect physicochemical changes, rather than the direct introduction of non-native microbial taxa. Despite differences in soil nutrient composition and nitrogen inputs across fertilization regimes, plant biomass, flower number, and root biomass did not show significant variation. This indicates that changes in microbial composition were driven more by soil nutrient availability than by plant growth responses. These findings reinforce the role of fertilization in shaping soil and root microbiomes, reducing microbial complexity through altered soil properties and the introduction of livestock-associated microbes [4,5,17,20]. Our results are consistent with those of Tyrrell et al. [71], who reported that swine, bovine, and poultry manure negatively impacted soil and phyllosphere

microbiomes, leading to altered microbial communities and increased abundance of ARGs and mobile genetic elements (MGEs). These findings further underscore the grass phyllosphere as a reservoir for ARGs and MGEs, which can confer resistance to clinically relevant antimicrobials.

Cascading effects of microbiota alterations across trophic chain

We observed microbial overlap across the trophic chain, with particularly high overlap between soil and rhizosphere microbiomes, as well as between soil and earthworm microbiomes. These findings suggest that fertilization-induced changes in soil and rhizosphere microbiomes may also influence the microbiomes of associated detritivores, herbivores, and ground-nesting pollinators. This aligns with growing evidence of microbial transmission from soil through roots to plant shoots, and ultimately to pollinators via flower-pollinator or soil-pollinator interactions [72–74]. Consequently, disturbances to microbiomes from fertilization may trigger cascading effects throughout the trophic chain.

While it is often assumed that allochthonous communities displace indigenous ones, thereby altering community composition [4,5,70], our findings suggest an indirect effect on native microbial communities, likely mediated by fertilization-driven changes in soil nutrient conditions. However, we detected four pig slurry-associated genera—*Clostridium sensu stricto 1, Terrisporobacter, Turicibacter,* and *Romboutsia*—in multiple trophic compartments. Although the relative abundance of these genera was low, their repeated detection across compartments suggests their ability to establish in diverse environmental niches. These findings raise significant health concerns. Previous studies have shown that repeated pig slurry application increases the diversity and abundance of ARGs and mobile genetic elements (MGEs), indicating potential long-term ecological impacts [75]. Pig slurry-associated taxa carrying ARGs pose a risk of horizontal gene transfer across both environmental and host-associated microbiomes. Their presence in pollinators and small mammals—organisms interacting with various ecosystem components—further highlights their potential role as reservoirs or vectors of microbial and genetic material.

In this study, we did not observe the previously reported microbial overlap between flowers and bumblebees [37]. This absence of overlap may be attributed to land use-driven reductions in floral resource abundance and diversity, which could lead insect pollinators to modify their foraging behavior [76]. However, *Turicibacter*—a pig slurry-associated microbe—was detected in bumblebees, which may be explained by their ground-nesting behavior and direct contact with fertilized soil, facilitating microbial overlap between belowground and aboveground compartments. Flowers, visited by multiple pollinators, may act as microbial reservoirs and transmission hubs, potentially influencing

pollinator health through the spread of fertilizer-associated taxa [37,72–74]. Similarly, belowground animals, such as the common vole, ingest soil and plant-associated microbes, affecting their microbiomes and potentially serving as reservoirs for antibiotic-resistant bacteria [8,25,36]. Fertilization-driven shifts in microbial communities, reduced complexity, and microbial influx can compromise host health. Moreover, affected hosts could serve as reservoirs for microbes carrying ARGs, further aiding their dissemination [6–8]. The potential for fertilizer-driven spread of ARGs and MGEs through trophic interactions in grasslands is a significant concern.

Microbial overlap along the trophic chain may suggest the potential for transmission across successive levels, highlighting the need to understand these dynamics to fully assess the impact of fertilization on grassland ecosystems, host organisms, and human health within a One Health framework [16]. Understanding microbial transmission dynamics between trophic compartments and fertilizers remains a major challenge, shaped by complex interactions involving environmental conditions, host physiology, trophic relationships, and microbial resilience. Definitive evidence would require controlled indoor studies using radioactively or fluorescently labeled microbes—an approach not feasible in field settings. Nonetheless, our 16S rRNA amplicon sequencing provides strong indications of potential transmission pathways influencing microbiomes across interconnected trophic compartments and lays the foundation for future controlled experiments to track marked microbes. Interpreting these transmissions requires considering host specificity and the effects of introduced or displaced microbial communities, emphasizing the need for further research.

In summary, fertilization—especially intensive pig slurry application—significantly alters microbiomes across grassland trophic compartments. The varied responses observed among compartments, along with partial cascading of microbial changes along the trophic chain, support the eco-holobiont framework [27], emphasizing the interconnectedness of microbial communities and their potential implications for both animal and human health.

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Author Contributions

Karoline Jetter (Investigation, Formal Analysis, Data curation, Writing—original draft, Writing—review & editing), Kunal Jani (Formal Analysis, Data curation, Writing—original draft, Writing—review&editing), KerstinWilhelm (Investigation), Ulrike Stehle (Investigation), Rostand R. Chamedjeu (Investigation), Christian U. Riedel (Conceptualization, Data curation, Writing— review & editing, Funding acquisition, Supervision), Lena Wilfert (Conceptualization, Data curation, Writing—review & editing, Funding acquisition, Supervision), Patrick Schäfer (Conceptualization, Data curation, Writing—review & editing, Funding acquisition, Supervision), Simone Sommer (Conceptualization, Data curation, Writing—review & editing, Funding acquisition, Supervision)

Conflict of interests

The authors declare no conflict of interest.

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Data availability

The datasets generated during and/or analysed during the current study conducted in cooperation with the Biodiversity Exploratories program (DFG Priority Program 1374) are publicly available on GitHub (https://github.com/kunaljaani/Fertilizationimpacts-microbiomes-along-the-grassland-trophic-chain/tree/9ed11ecadad8fd6b491dbffe77abdcfd9ff1747e/nutrientData) and on NCBI (Bioproject number: PRJNA1188490) [77,78].

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Supplemental Material

Fertilization impacts microbiomes along the grassland trophic chain

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Supplementary Methods

Sampling of focus specimens along the trophic chain

Soil, plant (red clover, *Trifolium pratense*), earthworm, bumblebee (*Bombus lapidarius*) and vole (*Microtus arvalis* and *Arvicola amphibius*) samples were collected in grassland ecosystems subjected to different fertilization regimes to investigate the effects of fertilization on microbiomes along the trophic chain (Figure S1B). All grassland sites except sites fertilized with pig slurry are part of the Biodiversity Exploratories framework (https://www.biodiversity-exploratories.de)[1]. The pig slurry fertilized sites were selected in cooperation with local farmers on the Swabian Alb (Figure S1B).

Soil, earthworms and plants were sampled together in an area of approximately 10cm x 10cm around a red clover stem. Soil was sampled by collecting the top layer (to a depth of 15 cm) from the 10x10x15 cm ditch and sifting through a 0.1 cm kitchen sieve to remove larger stones and roots. Samples of red clover *Trifolium pratense* were selected by visual inspection, choosing a healthy individual with at least one open flower at anthesis; large plants, usually characterized by large root systems, were excluded to facilitate root processing. Plant samples were collected by removing the plant with its roots from the ditch in order to retain the plant roots by carefully removing the bulk soil adhering to the roots. Plant height and number of flowers per plant were also recorded. Roots and flowers were separated from the aboveground biomass and all samples were kept on ice during transport to the laboratory for further processing. Root fresh weight was recorded and aboveground biomass was dried for 5-6 days at 60°C before measuring aboveground dry biomass. Due to the extremely dry climatic conditions during the summer of 2022, all earthworms were collected from the same 10x10x15cm ditch regardless of taxonomic affiliation. Earthworm feces were collected by placing the earthworms in a petri dish with a damp paper towel overnight and then releasing the earthworms. Bumblebees of the species *Bombus lapidarius* were captured at the field sites using a plastic container when visiting a flower inside or close to the site. Voles were captured using Sherman live traps with a bait containing a mixture of maltol solution, oat flakes and cereals, and an apple slice. The traps were modified according to Mori et al.[2] by attaching half a Tetra Pack carton with an entrance hole to the trap opening, which was then placed on a vole burrow. Traps were set in the late evening and checked early the next morning. If a vole was caught, its feces were collected in 400µl nucleic acid preservation (NAP) buffer[3] and the voles were released after recording their weight and sex. All samples were transported to the laboratory on ice for further processing and stored at -20°C until further analysis.

Sample processing

Plant samples were separated into root, flower and aboveground biomass samples. Root samples were separated into endosphere and rhizosphere samples (see Figure S1B). The rhizosphere was

washed from the roots by shaking the whole root in 25 ml sterile water, removing the roots and centrifuging the solution at 4000 × g for 15 min to obtain microbial cells. Endosphere samples were obtained by shaking the root in 30 ml of 0.1% (v/v) Triton X-100 in H_2O followed by washing off the Triton solution with 30 ml of sterile water. Roots were then air dried on sterile filter paper and cut into smaller pieces for extraction. Flowers were sonicated in 20 ml sterile PBS-Tween solution (1x, 0.15%) for 10 minutes to remove epiphytic microbes. Flower parts were removed with sterile tweezers and the solution was centrifuged at 3000 rpm for 10 minutes to pellet the microbial cells. Both root and flower samples were stored at -80°C until DNA extraction. Aboveground wet biomass was assessed before plant material was dried at 50°C for at least 7 days to determine aboveground dry weight.

Bumblebees were euthanized by freezing at -20°C and frozen bees were dissected under sterile conditions to extract the gut. Gut samples were frozen at -80°C until extraction. Vole and earthworm feces were stored at -20°C.

DNA extraction, library preparation and 16S rRNA gene amplicon sequencing

To analyze the microbial composition, DNA of root, earthworm feces, vole feces and soil samples were extracted using the *ZymoBIOMICS DNA Miniprep* kit (Zymo Research Europe, Germany), whereas the *ZymoBIOMICS DNA Microprep* kit was used to extract DNA of flower and bumblebee gut samples. All samples were extracted following the manufacturer's instructions and subjected to an initial bead beating step using ceramic beads provided by the kit to mechanically lyse bacterial cells. To break open the roots for the endosphere samples five steel beads (2.4 mm, Bio-Budget Technologies, Germany) were additionally added to each sample. Bead beating was carried out using a SpeedMill PLUS (Analytik Jena, Germany) running two 3-minute cycles of bead-beating interrupted by a 3 -minute break.

To assess the bacterial composition, the hypervariable V4 region of the 16S rRNA gene was amplified in a two-step polymerase-chain reaction (PCR) using the primers 515 F (5'-GTGCCAGCMGCCGCGGTAA-

3') and 806 R (5'-GGACTACHVGGGTWTCTAAT-3')[4]. To avoid amplifying high amounts of chloroplast and mitochondrial DNA in plant (endosphere and flower) and bumblebee gut samples, 10µM peptide nucleic acid (PNA)-DNA clamps (mPNA and pPNA in plant samples and mPNAs in bumblebee samples) were added to the first PCR reaction[5]. The PCR regime for these samples included one more annealing step for 30 seconds at 78°C after denaturation within the 30 cycles. PCR success for all samples was confirmed by agarose gel electrophoresis. Appended adapters for forward (CS1-515F) and reverse (CS2-806R) primers were used in order to construct the library using Standard Bio Tools chemistry (Access Array System for Illumina Sequencing Systems, Standard BioTools, USA)[6–8]. The second PCR reaction was performed to attach individual barcodes and adapters used for illumina sequencing. To account for potential sequencing artifices arising from the DNA extraction, a positive control mock community (Zymo Research Europe, Germany), as well as extraction blanks and PCR-negative controls, were also included as a part of the in-house Illumina Miseq sequencing run at the Institute of Evolutionary Ecology and Conservation Genomics at Ulm University in Germany.

Soil nutrient assessment by electro-ultrafiltration

EUF separates nutrients into two fractions based on different water pressures and membrane types: the first fraction represents nutrients immediately available to plants, and the second contains nutrients that are deliverable over time. For our analysis, we combined both the immediately available and deliverable fractions to calculate the total amount of nutrients for each site. We used the information of the content of eight macronutrients (P, K, S, N, nitrate, Ca, Mg, B) and four micronutrients (Mn, Zn, Cu, Fe) as well as the sodium content in soil samples for subsequent analyses.

Bioinformatic sequence processing

Sequence processing was conducted on Qiime2 (version 2022.8) using the DADA2 pipeline which incorporates primer removal, denoising, chimera removal and merging of paired-end reads[9,10]. Given the high number of samples and the samples being sequenced on two different runs, the denoising steps were conducted for each run separately. Files of the representative sequences, metadata files

and the dada2 output table were merged before taxonomical assignment. For the first run forward reads were trimmed at 30 and 240 bp and reverse reads were trimmed at 30 and 215bp. For the second run, forward reads were trimmed at 30 and 240bp, reverse reads were trimmed at 30 and 220bp. For taxonomical assignment the SILVA 138-99 database[11] using the q2-feature classifier classify-sklearn (version 0.24.1)[12] in QIIME2 was used and run on the combined dataset of both sequencing runs. A phylogenetic tree was constructed after all ASVs were aligned with mafft[13] (via q2-alignment) using fasttree[14] (via q2-phylogeny). Using the software Dendroscope (version 3.8.4)[15], the unrooted phylogenetic tree was re-rooted using an uncultured archaeal sequence (accession number KT433146.1). The rooted phylogenetic tree and the biom table, both generated in Qiime2, together with the metadata file were imported into R using the phyloseq package[16] and a phyloseq object was created to conduct all statistical analysis using the R language[17] within the R Studio interface (version 4.3.2, Posit team (2023). The data processing of the phyloseq object in R precluded filtering of unassigned taxa as well as taxa assigned to chloroplast and mitochondria on family, class or order level and non-bacterial sequences. It was followed by filtering of samples by retaining the samples with non-zero reads.

Supplementary Results

Fertilization affects microbial composition of different trophic compartments

The fertilizer effects on microbial composition were more prominent in all trophic compartments at the genus level. Across belowground compartments, the most prominent differences in fertilized sites compared to control sites were lower abundances in *Bacillus* and higher abundances in *Pseudomonas* (Figure S8). In soil, the predominant genera at control sites were *Bacillus* and *Rokubacteriales* whereas *Gaiella* was the dominant genus in fertilized sites. Although *Rokubacteriales* were one of the dominating genera in biogas-fertilized and cow/horse manure-fertilized as well as control sites, they

were present only in lower abundances in pig slurry-fertilized sites. Despite differences in abundance, *Pseudomonas* was the predominant genus in the rhizosphere in all fertilization regimes and in the endosphere in pig slurry-fertilized sites. *Kineosporia,* which dominated the composition of the endosphere in control, biogas digestate and cow/horse manure-fertilized sites, was far less abundant under pig slurry fertilization. *Flavobacterium* and *Bacillus* dominated the earthworm composition and *Solirubrobacter* were more abundant in fertilized sites as compared to control sites. Microbial composition in voles was dominated by *Ruminococcus* and two *Clostridia* groups in all treatments. *Gastranaerophilales* were present only in fertilized sites while *Roseburia* and *Acetatifactor* were less abundant in fertilized sites. In flower compartments, *Massillia* and *Ralstonia* were dominant in samples collected on biogas and pig slurry-fertilized sites, while *Duganella,* which dominated samples from control sites, were less abundant in fertilized sites. In bumblebees, where *Gilliamella* were most abundant in all fertilization regimes, *Pseudomonas* were more abundant and *Lactobacillus* were less abundant on fertilized sites compared to control sites. Genera dominating pig slurry composition were detected in all the compartments across the trophic chain with relative abundances between 1% and 5%, so these analyses suggest an indirect effect of fertilization on the microbiome compositions of the different compartments.

Table S1: Sample size distribution of the study. Number of samples included in the microbiome analyses in relation to the seven different trophic compartments and fertilization regimes. Samples marked with an asterisk (*) represent pools of fecal samples of one species due to low volume of material collected at one sampling site. All other samples represent individual non-pooled samples. Numbers in brackets indicate sample sizes per plot.

	Control	Biogas digestate	Cow/horse manure	Pig slurry	total
Soil	24 (4/4/4/4/4/4)	24 (4/4/4/4/4/4)	20 (4/4/4/4/4)	24 (4/4/4/4/4)	92
Rhizosphere (*Trifolium pratense*)	24 (4/4/4/4/4/4)	24 (4/4/4/4/4/4)	20 (4/4/4/4/4)	24 (4/4/4/4/4)	92
Endosphere (*Trifolium pratense*)	24 (4/4/4/4/4/4)	24 (4/4/4/4/4/4)	20 (4/4/4/4/4)	24 (4/4/4/4/4)	92
Earthworm	6* (1/1/1/1/1/1)	6* (1/1/1/1/1/1)	4* (1/1/1/1)	6* (1/1/1/1/1/1)	22*
Vole (*Microtus arvalis, Arvicola amphibious)*	5 (4/1)	16 (3/1/7/1/1/3)	25 (6/1/3/2/6/7)	3 (2/1)	49
Flower (*Trifolium pratense*)	24 (4/4/4/4/4/4)	24 (4/4/4/4/4/4)	20 (4/4/4/4/4)	24 (4/4/4/4/4)	92
Bumblebee *(Bombus lapidarius)*	18 (2/3/4/3/3/3)	13 (1/4/3/3/2)	16 (4/4/2/4/2)	4 (4)	51
Pig Slurry	0	0	0	4 (1/1/1/1)	4
Total	125	131	125	109	494

Table S2: Statistical results assessing the differences in alpha diversity (Shannon index) between the four different fertilization regimes within the seven different trophic compartments. Indicated are p-values of ANOVAs and post-hoc Tukey's HSD pairwise comparisons. Significant values are marked in bold.

Comparison	Soil	Rhizosphere	Endosphere	Vole	Earthworm	Flower	Bee
Overall	**0.029**	**0.001**	0.145	**0.001**	0.107	0.124	0.473
Control-pig	0.075	**0.001**	-	0.091	-	-	-
Control-biogas	0.981	0.987	-	**0.001**	-	-	-
Control-cow	0.959	0.060	-	**0.003**	-	-	-
Pig-biogas	**0.029**	**0.001**	-	0.881	-	-	-
Pig-cow	0.262	**0.049**	-	0.999	-	-	-
Biogas-cow	0.826	**0.026**	-	0.549	-	-	-

Table S3: Statistical results assessing differences in beta diversity (Bray-Curtis distances) between the four different fertilization regimes within the seven different trophic compartments. P-values are given for PERMANOVA and pairwise PERMANOVA comparisons. Significant values are marked in bold.

Comparison	All	Soil	Rhizo-sphere	Endo-sphere	Vole	Earth-worm	Flower	Bee
Overall	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.005**	**0.001**	**0.001**
Control-pig	**0.001**	**0.001**	**0.001**	**0.001**	0.656	**0.021**	**0.001**	0.229
Control-biogas	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	0.299	**0.001**	**0.023**
Control-cow	**0.001**	**0.001**	**0.001**	**0.002**	**0.001**	**0.005**	**0.001**	0.144
Pig-biogas	**0.001**	**0.005**	**0.001**	**0.007**	**0.003**	0.134	**0.002**	**0.005**
Pig-cow	**0.001**	**0.005**	**0.001**	**0.001**	**0.005**	0.121	**0.002**	0.37
Biogas-cow	**0.003**	0.153	**0.009**	**0.01**	**0.002**	0.052	**0.015**	**0.001**

Table S4: Statistical results assessing differences in nitrogen input and plant parameters between the different fertilization regimes. P-values are given for Kruskal-Wallis and posthoc pairwise Wilcoxon tests. Significant values are marked in bold.

Comparison	Nitrogen input	Aboveground biomass	Root fresh weigh	Plant height	Number of flowers
Overall	**0.001**	0.200	**0.023**	**0.001**	0.610
Control-pig	**0.010**	0.398	0.096	**0.001**	1.000
Control-biogas	**0.006**	0.301	1.000	**0.001**	1.000
Control-cow	**0.013**	1.000	**0.046**	1.000	1.000
Pig-biogas	**0.017**	1.000	0.330	1.000	1.000
Pig-cow	**0.010**	1.000	1.000	**0.001**	1.000
Biogas-cow	**0.010**	1.000	0.271	**0.003**	1.000

Table S5: Resilient and responding identifiable genera and ASVs of the different compartments. Genera have been filtered for unidentifiable taxa, that were not assigned until genus level or uncultured. Responding taxa are split for the paired comparison (all treatments compared to each other) and the contrasting comparison (all treatments against the control).

	Resilient	**Responder**	
		Contrasting	**Paired**
Soil	47 (110 ASVs)	9 genera (30 ASVs)	3 genera (9 ASVs)
Rhizosphere	62 (128 ASVs)	6 genera (21 ASVs)	1 genus (6 ASVs)
Endosphere	99 (228 ASVs)	17 genera (54 ASVs)	3 genera (9 ASVs)
Earthworm	67 (173 ASVs)	3 genera (9 ASVs)	1 Genus (3 ASVs)
Vole	15 (138 ASVs)	11 genera (51 ASVs)	8 Genera (39 ASVs)
Flower	12 (24 ASVs)	5 genera (15 ASVs)	0
Bumblebee	8 (19 ASVs)	18 genera (57 ASVs)	16 genera (48 ASVs)

Table S6: Set of interactions to decipher exchange of microbes across the trophic chain. Compartments soil, rhizosphere or endosphere were considered as potential source while key target compartments were endosphere, flowers, earthworms and voles.

	Source			
	Soil	Rhizosphere	Endosphere	Flower
Sink	Rhizosphere	Endosphere	Earthworm	Bumblebee
	Earthworm	Earthworm	Vole	
	Vole	Vole		
	Flower			

Table S7: Statistical results assessing differences in beta diversity (Bray-Curtis distances) between the seven different trophic compartments across all fertilization regimes using the refined dataset containing only resilient and responding genera. Indicated are p-values of Pairwise PERMANOVA comparisons. Significant values are marked in bold.

	Soil	Rhizosphere	Endosphere	Earthworms	Voles	Flowers
Rhizosphere	**0.001**					
Endosphere	**0.001**	**0.001**				
Earthworm	**0.001**	**0.001**	**0.001**			
Vole	**0.001**	**0.001**	**0.001**	**0.001**		
Flower	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	
Bumblebee	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**

Table S8: Statistical results assessing differences in beta diversity (Bray-Curtis) between the four different fertilization regimes across all trophic compartments using the refined dataset containing only resilient and responding genera. Indicated are p-values of Pairwise PERMANOVA comparisons. Significant values are marked in bold.

	Control	Biogas digestate	Cow/horse manure
Biogas digestate	**0.001**		
Cow/horse manure	**0.001**	**0.007**	
Pig slurry	**0.001**	**0.001**	**0.001**

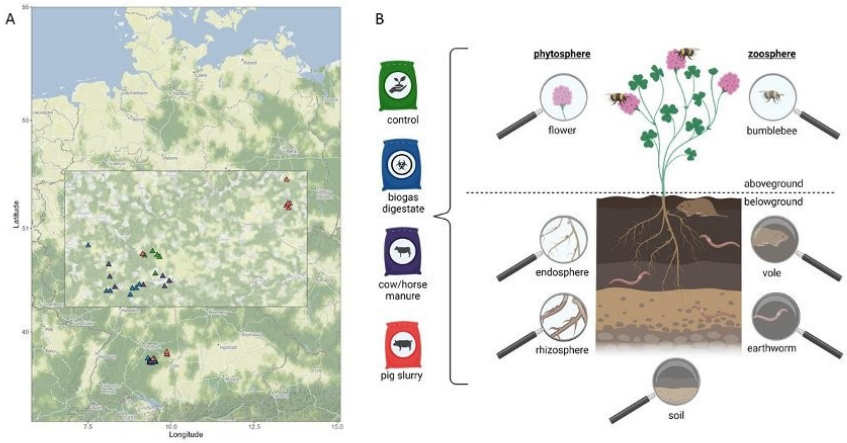

Figure S1: **Study design.** (A) Map of sampling sites located on the Swabian Alb in Baden-Württemberg, Germany, subjected to four different fertilization regimes (green: minimally fertilized control sites, blue: sites fertilized with biogas digestate, purple: sites fertilized with cow/horse manure, pink: sites fertilized with pig slurry). Map was created using R. (B) Study set up with seven different compartments of the trophic chain: soil, rhizosphere, root endosphere and flower (*Trifolium pratense)*, earthworm, vole (*Microtus arvalis, Arvicola amphibious*) and bumblebee (*Bombus lapidarius*) as well as the four different fertilization regimes: minimally fertilized control, biogas digestate fertilization, cow/horse manure fertilization and pig slurry fertilization. Compartments of the trophic chain can be grouped either in aboveground (flower and bumblebee) and belowground (soil, rhizosphere, endosphere, vole and earthworm) or into phytosphere compartments (rhizosphere, endosphere and flower) and zoosphere compartments (earthworm, vole, bumblebee). Figure created with BioRender.com.

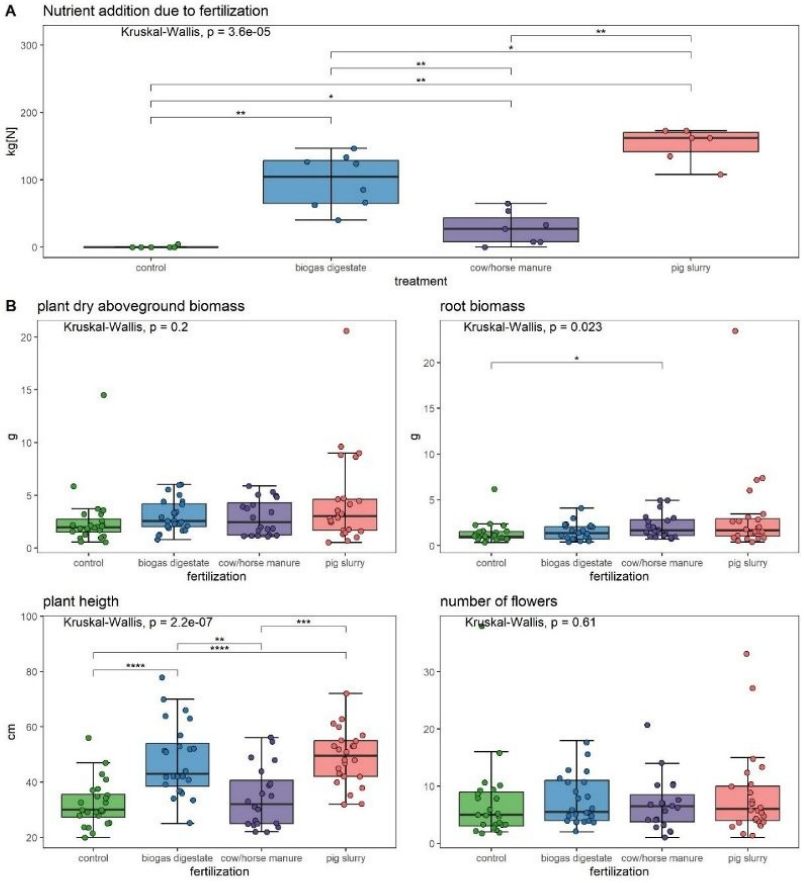

Figure S2: Nitrogen addition (A) and plant growth parameters (B). Amounts of nitrogen added to the respective field sites (A) was based on information from the land owners and the mean value of the sampling year and the last 4 years before sampling (2018-2022) was plotted. Organic fertilizers were converted into amount of nitrogen using standardized values from the LWK Nordrhein-Westfalen (2014) and the LWK Baden-Württemberg (2012), which are consistently used within the Biodiversity Exploratories' framework. Plant growth parameters aboveground dry biomass, root fresh biomass, plant height and number of flowers (B) were assessed for every plant collected from the field. In order to assess differences between the four fertilization regimes, Kruskal-Wallis tests followed by pairwise Wilcoxon post hoc tests were used.

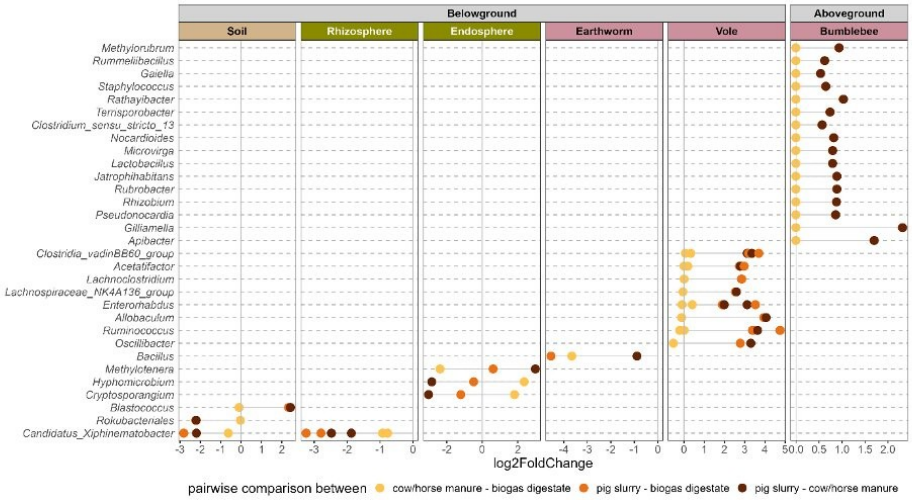

Figure S3: Bacterial genera with abundance changes in response to fertilization in different trophic compartments. Pairwise comparisons were made between cow and horse manure/biogas digestate (yellow), between sites fertilized with pig slurry/biogas digestate (orange) and between sites fertilized with pig slurry/cow and horse manure (brown) using ANCOMBC2. Flowers did not have any identifiable genera being significantly different for the tested comparisons and are hence not shown. Positive values of log2fold change represent genera that are more abundant in the second partner in the comparison; negative values represent genera that are more abundant in the first partner in the comparison.

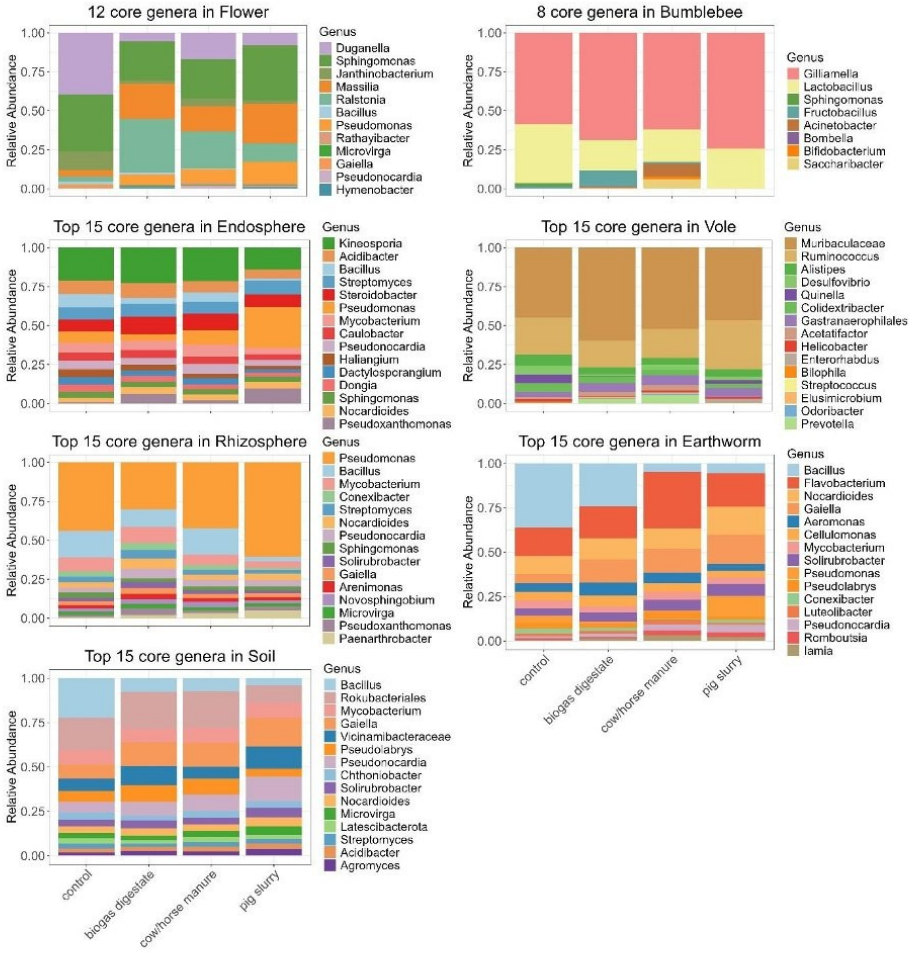

Figure S4: Composition bar charts of the core genera in the different compartments along the trophic chain. Indicated are the 15 most abundant core genera in soil, rhizosphere, endosphere, earthworms and voles, as well as the 12 core genera in flowers and 8 core genera in bumblebees in samples collected from sites subjected to the four different fertilization regimes (minimally fertilized control sites, biogas digestate fertilized sites, cow/horse manure fertilized sites and pig slurry fertilized sites).

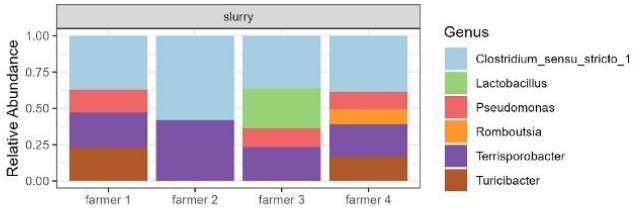

Figure S5: **Bacterial composition of the pig slurry.** Bacterial composition of pig slurry samples obtained from farm sites before application to the field indicating genera with a minimum abundance of 5%. Data represents individual samples.

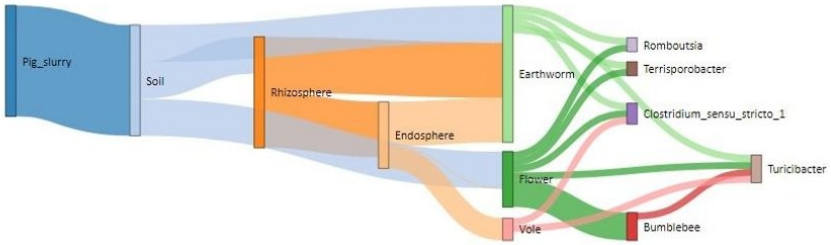

Figure S6: **Sourcetracker analysis showing the proportions of bacterial genera from pig slurry shared between compartments along the trophic chain.** Pig slurry was the primary source to all subsequent compartments, i.e. soil, rhizosphere, endosphere, while the main target compartments were earthworms, voles and flowers.

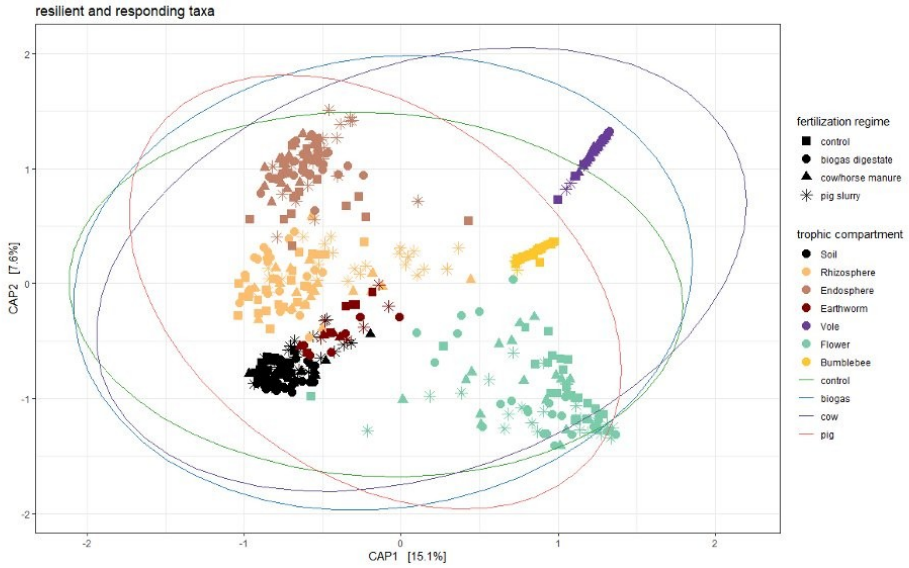

Figure S7: Two-dimensional illustration of the refined dataset containing resilient and responder taxa identified by CAP analysis. The different trophic compartments are indicated by colors and the fertilization regimes by different shapes.

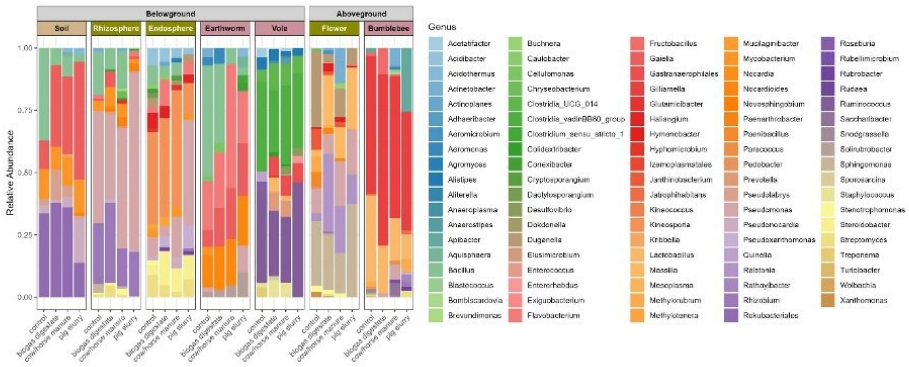

Figure S8: Bacterial composition of the different compartments along the trophic chain. Indicated are genera with a minimum abundance of 5%. Phytosphere compartments are marked in green, zoosphere compartments are marked in pink, soil is marked in brown. Data was pooled for plotting based on the fertilization regime within each compartment. Every fertilization regime contains up to 6 plots with 4 samples each, i.e. each bar represents a pool of up to 24 samples (see Table S1 for sample sizes).

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Chapter 3: Effects of Pig Slurry Fertilization History on Grassland Microbiomes: Insights from Field and Mesocosm Experiments

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Effects of Pig Slurry Fertilization History on Grassland Microbiomes: Insights from Field and Mesocosm Experiments

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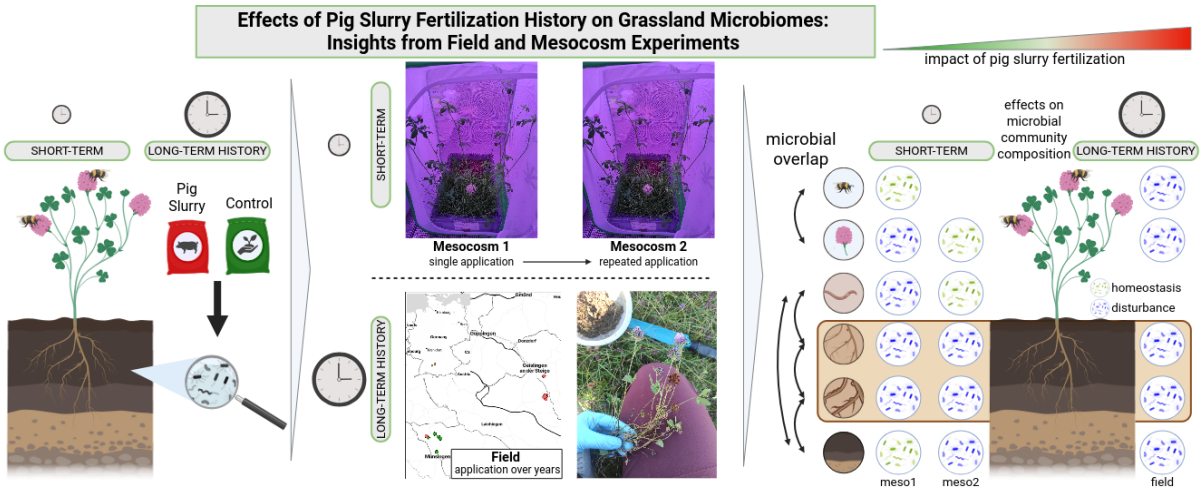
Keywords: pig slurry, fertilization, microbiome, trophic chain, mesocosm, long-term legacy effect, short-term effect

Abstract

Land use, particularly fertilization, alters species communities and ecological interactions in grassland ecosystems. While long-term pig slurry application is known to affect microbial communities across different compartments of the trophic chain, the short-term effects of organic fertilization have typically been studied in isolated individual compartments. In this study, we used 16S rRNA gene amplicon sequencing to examine microbial communities from grassland sites with a history of pig slurry fertilization and laboratory mesocosms simulating repeated short-term applications. We assessed both immediate and legacy effects on soil-plant-animal compartments across the trophic chain, using Trifolium pratense as a focal plant species, examining below-ground (soil, plant rhizosphere and endosphere, earthworms) and above-ground compartments (flowers and bumblebees). Root compartments, i.e., the rhizosphere and endosphere, were similarly affected by both short-term and long-term pig slurry application, whereas the other compartments were mainly affected by long-term legacy effects in the field. Laboratory mesocosm setups, representing the effects of repeated individual fertilization events, successfully simulated grassland ecosystems, including key ecological interactions. These experiments highlight the need for complex systems with repeated fertilizer applications to accurately replicate and reconstruct long-term field effects of fertilization history. This study underscores the importance of a holistic approach to evaluate anthropogenic practices like fertilization on ecosystems and highlights both legacy and immediate impacts of intensive pig slurry application on grassland microbial communities, with broader implications for ecosystem functioning and health.

Graphical abstract



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1. Introduction

Agricultural grassland is primarily used to produce fodder components, such as hay and silage, for livestock. The productivity of these grassland ecosystems in terms of hay and silage yield, is directly correlated with fertilization intensity (Seabloom et al., 2021). Organic fertilizers are considered more sustainable than inorganic alternatives, as they supply essential nutrients for plant growth and development, enhancing soil organic carbon and nutrient content, while being less detrimental to plant biodiversity than inorganic fertilizers (He et al., 2022; Shi et al., 2024; Villa-Galaviz et al., 2023). However, organic fertilizers – particularly animal manure – can significantly alter soil pH and nutrient composition, which in turn affects microbial community composition (Bebber and Richards, 2022; Jetter et al., 2024; Wu et al., 2020).

Moreover, organic fertilizers are often enriched with bacteria that can potentially affect the complexity of microbial networks, especially under intensified land use (Gaube et al., 2020; Ma et al., 2021). In addition, they may carry antimicrobial resistance genes which may in turn influence microbial community functions (Yu et al., 2024). This diversity of effects on microbiomes is observed across different compartments including soil, plant roots, leaves, flowers and even animal guts, and can lead to changes in host health and overall ecosystem functioning (Barko et al., 2018; Engel et al., 2016; Hartmann and Six, 2023; Singh et al., 2020; Trivedi et al., 2020). Disturbances in nutrient allocation and pathogen defense (Barko et al., 2018; Tong et al., 2024; Yang et al., 2024) through the disruption of microbial communities (e.g., dysbiosis), can have severe consequences for both hosts and the environment.

Land use in grasslands typically reflects long-term management practices, and extended fertilization often leads to increased soil microbial biomass and can also affect microbial diversity and community composition (Bebber and Richards, 2022; Jetter et al., 2024; Luo et al., 2015; Mora-Salguero et al., 2025). In our previous work analyzing microbial communities associated with plant and animal compartments across the trophic chain in grassland ecosystems, we found that long-term application of pig slurry has significantly host-dependent influences on microbial community compositions in different trophic compartments (Jetter et al., 2024). Such long-term management practices, and the associated fertilization history, impose legacy effects on microbial communities by decreasing soil microbial diversity and altering community composition (Zhang et al., 2018). However, the impact of fertilization is not necessarily limited to legacy effects of long-term application. For example, Suleiman et al. (2016) observed reduced microbial diversity just days after fertilization. Similarly, short-term applications of organic fertilizers have been shown to affect the microbial and functional diversity of soil communities (Kraut-Cohen et al., 2023; Lazcano et al., 2013).

To date, studies on the short-term effects of organic fertilization have focused on isolated soil or root-associated compartments, often overlooking trophic relationships. However, trophic interactions are essential for overall ecosystem function and the potential microbial exchange between different trophic compartments. Shifts in microbial communities within one compartment can propagate across the trophic chain, influencing higher trophic levels (Jetter et al., 2024; Liu et al., 2019). Plant roots, for example, excrete nutrients to recruit beneficial soil microbes (Eichmann et al., 2021; Trivedi et al., 2020), while herbivores and detritivores consume soil organic matter and plant parts, thus ingesting plant- and soil-associated microbes (Liu et al., 2018; Zhu et al., 2021). Pollinators, by visiting flowers from different plants, facilitate microbial exchange, with flowers acting as both reservoirs and transmission sites for microbial communities (Keller et al., 2021; Vannette, 2020). These examples underscore the need for a broader understanding of microbial dynamics across the trophic chain. Studying eco-holobionts (Singh et al., 2020) requires analyzing compartments across the trophic chain to evaluate both the short-term and long-term effects of fertilization, ultimately proving a comprehensive understanding of its impact on grassland ecosystems.

Building on our previous findings that pig slurry fertilization significantly impacts microbial communities across the trophic chain (Jetter et al., 2024), we conducted field sampling and established lab-scale mesocosms to distinguish between the short-term effects of a single fertilization and the impact of repeated pig slurry application, considering the role of fertilization history across various trophic compartments in grassland ecosystems. We sampled before and after fertilization events in both lab-scale mesocosm setups (with no fertilization history) and pig-slurry-fertilized field sites (with a long fertilization history) to understand: 1) the short-term effects of pig slurry application on microbial communities across different trophic compartments, 2) the impact of fertilization history on short-term effects of pig slurry on microbial communities, and 3) whether mesocosms can simulate and validate fertilization effects in grassland ecosystems. We hypothesized that a single pig slurry fertilization would immediately affect microbiomes in below-ground and lower trophic compartments (i.e., soil and roots), that any short-term effects would be affected by the fertilization history, and that mesocosms with repeated fertilizer application could simulate long-term fertilization legacy effects.

While single pig slurry applications in mesocosms affected root-associated communities, these changes were less pronounced than those observed in samples from field sites with long-term fertilization history. Similarly, soil and animal-associated microbial communities were not significantly altered by short-term fertilization, but showed strong responses under a long-term history of pig slurry fertilization in the field. Our results demonstrate that the effects of long-term fertilization history outweigh those of individual short-term fertilization events on microbial communities. Complex mesocosm setups are effective for investigating short-term fertilization impacts on microbial

communities, particularly those associated with plant roots and flowers, and have the potential to simulate long-term field conditions after repeated fertilization.

2. Materials and Methods

Following the setup of our previous study (Jetter et al., 2024), we collected samples from the field, representing both animal and plant compartments across the trophic chain in grassland ecosystems subjected to two fertilization regimes: an unfertilized control (C) and intensive fertilization with pig slurry (PS). In addition, we set up laboratory mesocosms to develop an experimental grassland system for validation and comparison. Field samples are subjected to long-term management and thereby experience a long-term history of fertilization, while mesocosm experiments have no or – due to repeated fertilization - only a limited history of fertilization. Animal compartments included bumblebees (*Bombus lapidarius* in the field and *Bombus terrestris* in the mesocosms) and earthworms (*Lumbricus terrestris* in the mesocosms), while plant compartments included the rhizosphere, endosphere, and flowers of *Trifolium pratense*. The sampled compartments were consistent across all datasets (field, mesocosm 1 (meso1), mesocosm 2 (meso2)), except for bumblebees, which were not available in the second indoor experiment (meso 2) (Figure 1), and earthworms, which were not available in the field. All three datasets include samples collected at two time points: before and after an immediate fertilization and mowing event, referred to as sampling time point 1 (TP1) and sampling time point 2 (TP2) (Figure 1).

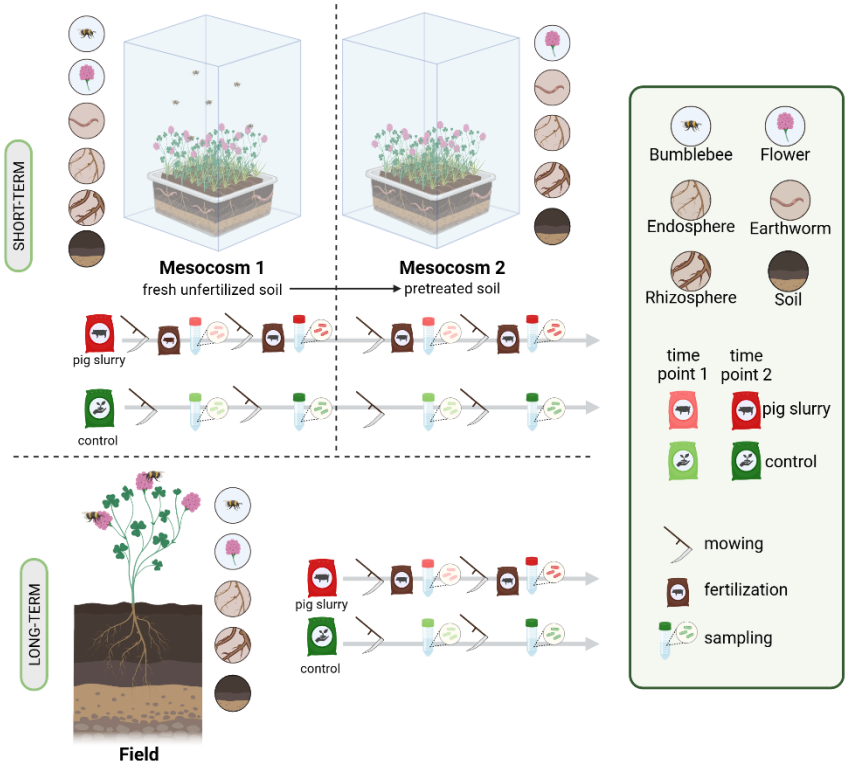

Figure1: Study Setup. Data were collected from two indoor mesocosm experiments and field sampling. Sampling occurred at two time points, before and after a fertilization and mowing event, with samples taken from sites or setups either fertilized with pig slurry or from unfertilized control sites. The two experimental setups represent short-term effects, with the mesocosm setup involving soil that had not been previously fertilized but received four fertilizer applications during the course of the experiment while the field soil has a long pig slurry fertilization history. Figure created with BioRender.com.

2.1. Field sampling

Field samples were collected on agriculturally used grassland sites on the Swabian Alb in Baden-Württemberg, Germany, between May and July 2022. These sites have been managed in the same way for the past 20 years. Sites were either unfertilized (i.e., former military training sites that are minimally managed by grazing flocks of sheep) or intensively fertilized with pig slurry up to 3 times per year. Control sites were part of the Biodiversity Exploratories research framework (https://www.biodiversity-exploratories.de) (Fischer et al., 2010) and pig slurry fertilized sites were selected in cooperation with local farmers. In total, 12 grassland sites were included in this study, six unfertilized control sites and six sites fertilized with pig slurry. Sampling of compartments and sample processing was conducted as previously described in Jetter et al. (2024). In short, four selected plants per site were uprooted and soil samples were collected from the top 15cm of the ditch (TP1). Roots

and flowers were separated from the remaining aboveground plant parts. Bumblebees were sampled while foraging on flowers at or close to the sampling sites and earthworm communities were collected from the ditch of the plant sampling, irrespective of taxonomy. Once fertilization and mowing were conducted by the farmer and plants had regrown (roughly 3-4 weeks later), the sites were sampled again in the same manner (TP2).

2.2. Experimental set up of mesocosms

Mesocosm experiments were set up in a laboratory at the University of Ulm (Figure 1). For meso1, unfertilized soil excavated at the Botanical Garden at the University of Ulm was used to fill eight plastic boxes (60 cm x 40 cm x 15 cm) in which eight *Trifolium pratense* plants as well as a community of common grasses (*Festuca rubra, Lolium perenne, Poa pratensis*) were planted and 40 earthworms (*Lumbricus terrestris*, Superwurm GmbH, Düren, Germany) were released. Four out of eight boxes were initially fertilized with 250 ml of pig slurry (from cooperating farmers on the Swabian Alb) diluted to 500 ml with water, equivalent to 10 m^3/ha) at the beginning of the setup, the other four boxes were left unfertilized. As soon as all clover plants of a box were flowering, a microcolony of 32 bumblebee workers (*Bombus terrestris*, Biobest, Westerlo, Belgium) was introduced to the box within a flight cage. One week after the introduction of bumblebees, all bumblebees, four plants of each box and all earthworms found while removing the plants were sampled (TP 1). The remaining clover plants and the grass were cut at about 3cm height to imitate mowing and pig slurry fertilized boxes received another fertilization treatment similar to the first one (with 250 ml of pig slurry, diluted to 500 ml in water). After re-flowering of the cut clover plants, a new microcolony was introduced into the flight cage and the remaining four clover plants, all remaining earthworms and all bumblebees were sampled one week after introduction of bumblebees (TP2).

In the second setup (meso2), 20 Trifolium pratense plants were individually sown into pots filled with soil re-used from the first mesocosm experiment, with each set receiving the respective fertilization treatment. Ten plants were grown in soil from pig slurry-treated boxes, while the other ten grew in soil from control boxes. Once the plants began flowering, those in the pig slurry treatment were fertilized with 20 ml of pig slurry (diluted to 50 ml in water, equivalent to meso 1), and earthworms were introduced one week later. 33 days after flowering, half of the plants were sampled (TP1), while the other half was cut to about 3 cm in height to simulate mowing, followed by a second fertilization with 20 ml of pig slurry. Another set of samples was taken 33 days after reflowering (TP2). Further details on the experimental setups are provided in the supplements.

2.3. Sample processing, DNA extraction, library preparation and 16S rRNA gene amplicon sequencing

In total, 705 samples were collected from the field and the two mesocosm experiments. Details on the sample sizes per compartment, sampling round and experiment are given in Table S1. Sample processing for all samples was conducted as previously described in Jetter et al. (2024). In short, root samples were separated into rhizosphere and endosphere by washing off the rhizosphere and sterilizing the roots. Flower microbes were dislocated from the plant biomass, frozen bumblebees were dissected to extract the gut and earthworm feces was collected after leaving them in a Petri dish to defecate overnight. All samples were frozen at -80°C (field) or -20°C (meso1 and meso2) until extractions.

Similar to sample preparation, DNA extraction, library construction and sequencing were also performed as previously described in (Jetter et al., 2024). In short, samples were extracted using commercial DNA extraction kits, the V4 region of the 16S rRNA gene was amplified in a two-step-PCR using the primers 515 F (5'-GTGCCAGCMGCCGCGGTAA3') and 806 R (5'-GGACTACHVGGGTWTCTAAT-3') as well as peptide nucleic acid (PNA)-DNA clamps to avoid amplifying high amounts of chloroplast and mitochondrial DNA. Constructed libraries were paired-end sequenced in four different runs on an in-house illumina MiSeq sequencing platform. Sequencing resulted in 18163328 reads and 54131 ASVs after filtering.

2.4. Soil nutrient content assessment

As previously described in Jetter et al. (2024), soil samples from the field and the first mesocosm experiment were analyzed for total concentrations of eight macronutrients (nitrate, organic nitrogen, phosphorus, potassium, sulfur, boron, magnesium, and calcium), sodium, and four micronutrients (iron, manganese, zinc, and copper). Briefly, for the field data, soil was pooled by plot and sampling time point, while in the first mesocosm experiment (meso1), soil was pooled by treatment at the first sampling time point. This resulted in 22 pooled samples for the field and 2 pooled samples for meso1, which were submitted to Bodengesundheitsdienst GmbH (https://www.bodengesundheitsdienst.de) for analysis via electro-ultrafiltration.

2.5. Bioinformatic sequence processing and statistical analyses

Bioinformatic processing of raw demultiplexed sequences of all three datasets was conducted as described in Jetter et al. (2024) using the DADA2 pipeline in the QIIME2 (version 2024.2) (Callahan et al., 2016; Evan Bolyen et al., 2019), which includes the removal of primers, denoising, removal of chimeras and merging of paired-end reads. Further details are provided in the supplementary

material. Further statistical analyses, filtering and graphics were performed in the R programming language using the R studio user interface (R Core Team, 2025).

To assess the effects of long-term and short-term pig slurry application on microbial communities associated with different hosts across the trophic chain, alpha diversity (Shannon index) and beta diversity (Bray-Curtis distances) were calculated separately for each compartment within the three datasets. Differences between sampling time points, treatments, and their interactions were evaluated using linear mixed effect models followed by emmeans tests with Holm-Bonferroni method p-value adjustment (Holm, 1979) for alpha diversity, and PERMANOVAs with pairwise PERMANOVA for beta diversity. These analyses were performed using the lme4, vegan and pairwise Adonis package in R (Bates et al., 2015; Martinez Arbizu, 2017; Oksanen et al., 2022).

The core microbial members of each compartment and dataset were determined separately for each fertilization and sampling time point (TP) combination (TP1-C, TP2-PS, TP2-C, TP2-PS) using the core function from the microbiome package (Lathi and Shetty, 2019), with detection threshold of 0.001 and a prevalence of 0.5. Differential abundance analysis on this core microbiota was performed using ANCOMBC2 across the three datasets (Lin et al., 2022; Lin and Peddada, 2020). Comparisons were made between control and pig slurry-treated samples at the first sampling time point (TP1), as well as between both fertilization treatments at the second sampling time point (TP2).

Redundancy analysis (RDA) based on Bray-Curtis distances was performed on the field soil microbial community to evaluate the influence of soil nutrient content on microbial composition. Available nutrients (P, K, S, N, NO3, Na, Ca, Mg, B, Mn, Zn, Cu, Fe) were used as explanatory variables. A forward selection model was constructed using the ordiR2step function from the vegan package (Oksanen et al., 2022) to identify nutrients that significantly contributed to variation in the microbial community. RDA models were then run using the ordinate function from the microViz package (Barnett et al., 2021), testing all nutrients together as well as macro- and micronutrients separately. All graphics were created in R using the ggplot2 package (Wickham, 2016).

3. Results

3.1. History of long-term fertilization with pig slurry affects the diversity of microbial communities

In this study we compared a dataset originating from grassland sampling (field) with a long history of fertilization to two indoor mesocosm experiments with no (meso1) or limited (meso2) history of fertilization containing samples from six different trophic compartments and two different treatments (C, control; PS, pig slurry). As shown in Figure 1, after an initial fertilization round in the field and

mesocosm experiments, samples were collected at two different time points (TPs), before (TP1) and after (TP2) a second fertilization event (median of days between PS fertilization and sampling; meso1: TP1:92 TP2: 43.5; meso2: TP1:32, TP2: 32; field: TP1: 49, TP2: 24; see Figure S1; control sites were also sampled at the respective dates), reflecting short-term effects. Mesocosm experiments were successive in that C or PS-fertilized soil from meso1 was re-used to set-up meso2. This allowed to at least partially reproduce PS fertilization history of grassland soil and, hence, to analyze PS effects on microbiome dynamics in ecosystems without PS application history and with a longer PS application history (see Figure 1).

Comparison of alpha diversity (Shannon index) in field sites across trophic compartments overall revealed an increase in diversity in soil and flower microbiomes between TP1 and TP2 (see Fig. 2). We detected a significant increase in soil microbial diversity at control field sites at TP2 (TP2-C) as compared to TP1-C ($p = 0.017$, Table S2). Rhizosphere samples collected from PS-fertilized field sites at TP1 (TP1-PS) showed significantly lower Shannon diversity as compared to TP1-C, as well as to samples from TP2 regardless of treatment (TP1-C vs. TP1-PS: $p = 0.008$; TP1-PS vs. TP2-C: $p = 0.003$; TP1-PS vs. TP2-PS: $p = 0.015$, Table S2). In flower samples, we observed significantly higher alpha diversity at TP2-C as compared to TP1-C sites ($p = 0.001$; Figure 2, Table S2). In contrast, no significant differences were detected in endosphere, earthworm or bumblebee samples (Figure 2, Figure S2, Table S2). Unlike the field samples, which showed significant changes in soil, rhizosphere, and flower compartments, PS fertilization had no significant effect on any compartment in either mesocosm experiment (Figure 2, Table S2), and sampling timepoint only significantly impacted endosphere samples in meso1 (TP1-PS vs-TP2-C: $p = 0.026$; TP1-PS vs. TP2-PS: $p = 0.009$, Table S2). The fact that PS-induced changes occurred exclusively in field samples with a long-term fertilization history suggests that PS fertilization exerts cumulative legacy effects on rhizosphere microbial diversity and that these effects might be amplified under field conditions. However, meso2 data from soil rhizosphere, and flowers showed diversity patterns similar to field samples suggesting that continuous mesocosm fertilization can reflect long-term history effects of PS application.

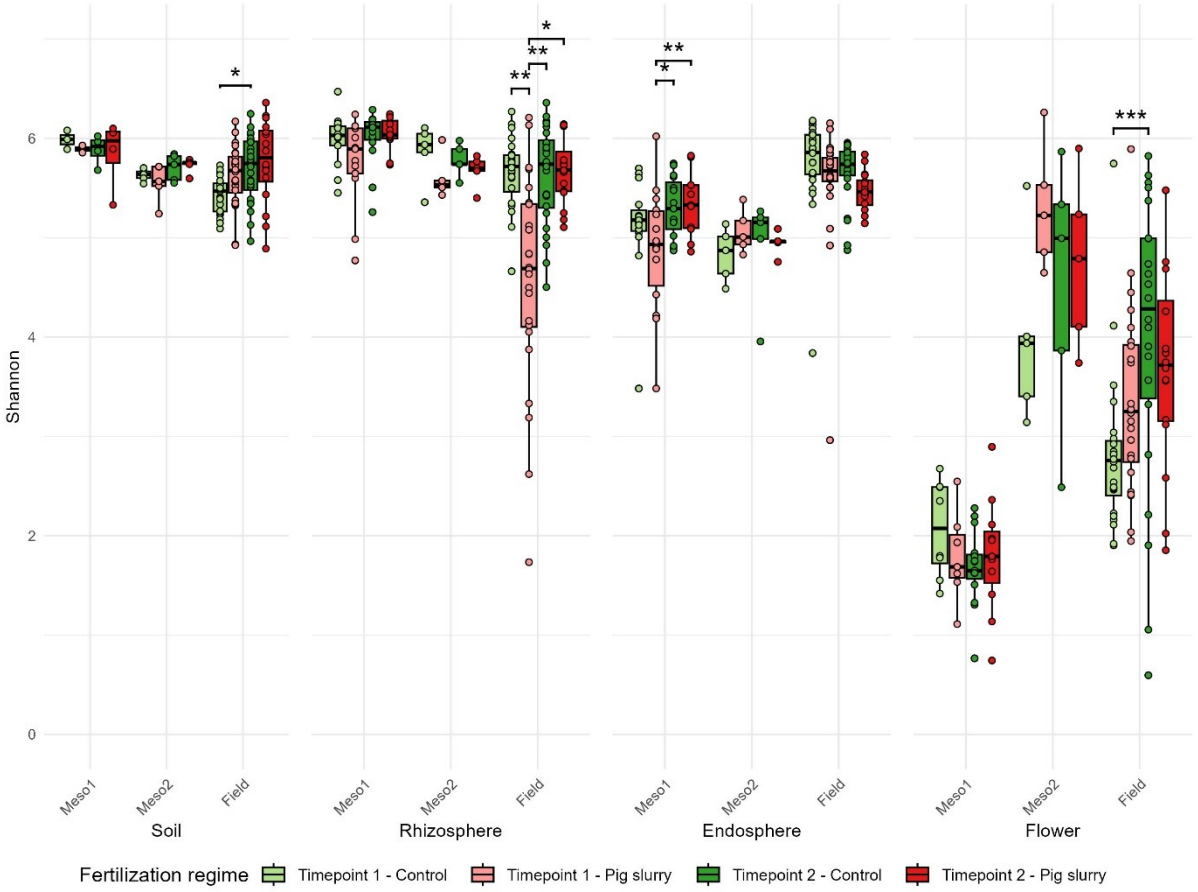

Figure 2: Alpha diversity of the different trophic compartments. Bars represent different set of samples, with Shannon index calculated for each dataset and compartment individually. Colours indicate control or pig slurry-fertilized samples, as well as the sampling round. Differences between samples were tested using a linear model followed by emmeans post hoc tests.

3.2. Long-term fertilization history as well as short-term application of pig slurry affect the composition of microbial communities

Next, to assess the impact of PS fertilization on the microbial composition of the different compartments, Bray Curtis distances were calculated. PS fertilization and sampling time point (TP) affected the composition of soil microbial communities of long-term fertilized field samples (Figure 3). (PERMANOVA: PS: $p<0.001$ ($R2=0.126$); TP: $p=0.032$ ($R2=0.021$)). Here, all pairwise comparisons of the interaction of fertilization and sampling TP (TP*PS) were significant except for the comparison between first and second sampling of PS-fertilized samples (TP1-PS vs TP2-PS) as well as first sampling of control and PS-fertilized samples (TP1-C vs TP1-PS) (Table S3). In both root compartments (rhizosphere and endosphere), microbial composition also differed according to fertilization, between sampling TPs, and due to their interaction in the field (PERMANOVAs: rhizosphere - field: PS: $p<0.001$ ($R2=0.078$); TP: $p<0.001$ ($R2=0.040$); TP*PS $p<0.001$ ($R2=0.029$); endosphere - field: PS: $p<0.001$

(R2=0.094); TP: p=0.001 (R2=0.021); TP*PS p00.048 (R2=0.011)) (Table S3). For both rhizosphere and endosphere, all pairwise comparisons of the interaction between fertilization and TP in the field samples were significant (Table S3). For flowers, both fertilization and sampling TP had significant effects on the microbial composition in field data as well (PERMANOVA: PS: p<0.001 (R2=0.046); TP: p<0.001 (R2=0.080); TP*PS p<0.001(R2=0.026)) (Table S3), with all pairwise comparisons between fertilization and TP combination being significant (Figure 3). In contrast, for bumblebees, only sampling TP showed a significant effect (PERMANOVA: TP: p=0.029, R2=0.036) (Table S3), while fertilization had no detectable impact (Figure S3). These results show that microbial community composition of soil and all plant compartments are impacted both by their fertilization history, as well as by a single PS fertilization application in field sites with a long-term fertilization history.

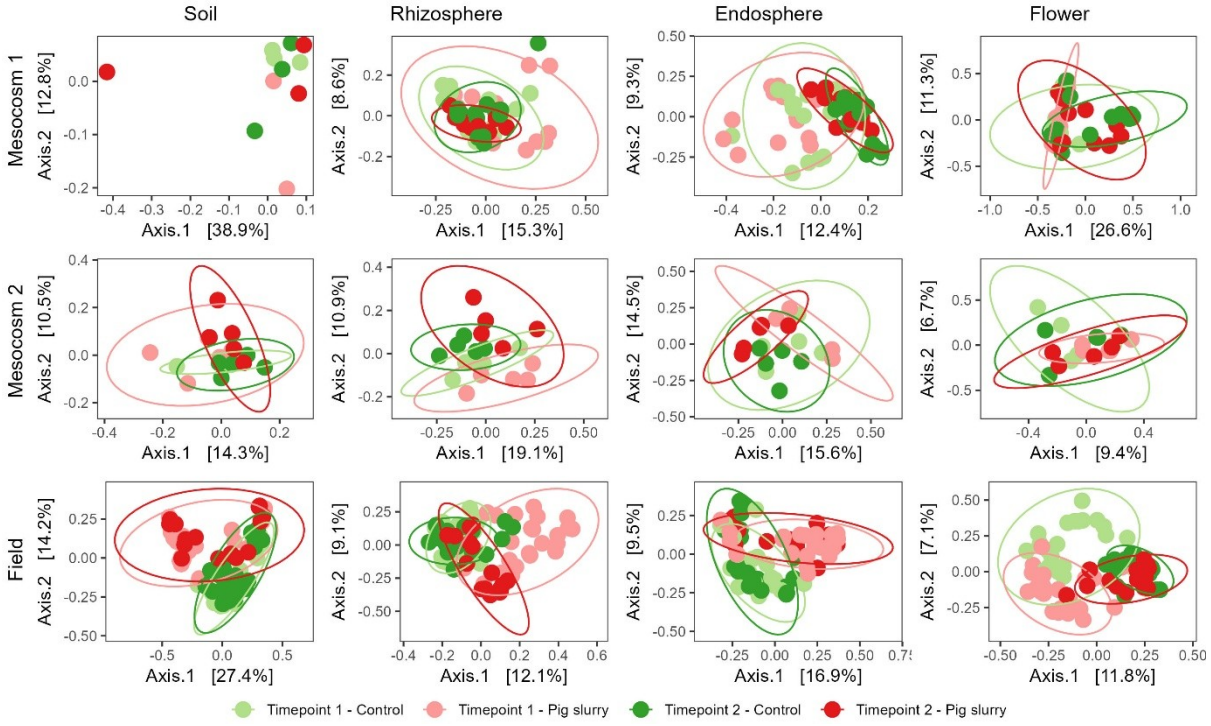

Figure 3: **Beta diversity based on Bray-Curtis distances in multidimensional scaling**. The four most impacted trophic compartments soil, rhizosphere, endosphere and flower are shown for the three different datasets. Colors represent control or pig slurry-fertilized samples, as well as the sampling round.

In the mesocosm experiments, in contrast, we found that while the first application of PS (meso1) had no significant effect, repeated PS fertilization (meso2) had an effect on the mesocosm soil samples (PERMANOVA: PS: p=0.010 (R2=0.070), Fig. 3). In both root compartments, fertilization and sampling TP affected microbial composition in both mesocosm experiments (PERMANOVA – rhizosphere: meso1: PS: p=0.001 (R2=0.034); TP: p<0.001 (R2=0.045); TP*PS: p=0.014 (R2=0.023); meso2: PS: p=0.001 (R2=0.104); TP: p=0.003 (R2=0.090); TP*PS: p=0.111 (R2=0.055); endosphere: meso1: PS:

p=0.001 (R2=0.028); TP: p<0.001 (R2=0.079); TP*PS: p=0.360 (R2=0.016); meso2: PS: p=0.013 (R2=0.080); TP: p=0.003 (R2=0.098); TP*PS: p=0.127 (R2=0.061) , Figure 3). In the rhizosphere, all pairwise comparisons except for TP1-PS vs TP2-C in meso1 and TP1-C – TP2-C in meso2 (Table S4 and Table S5) were significant, while in the endosphere all combinations except for TP1-C vs.TP1-PS and TP1-C vs TP2-PS in meso1, TP1-C vs TP1-PS in meso2 (Table S4 and Table S5) differed significantly. For flowers, differences in microbial composition according to fertilization and sampling TP were significant in meso1 (PERMANOVA: PS: p=0.004 (R2=0.044); TP: p=0.018 (R2=0.051); TP*PS p= 0.314 (R2=0.025), Figure 3), but not in meso2. Similarly, in earthworms, microbial composition was only affected by fertilization and sampling TP in meso1 (PERMANOVAs: PS: p=0.002 (R2=0.094); TP: p=0.002 (R2=0.216); TP*PS p=0.418 (R2=0.039)), but not in meso2. In meso1, pairwise comparisons of fertilization and sampling TP were significant for TP1-C vs TP2-C and TP1-C vs TP2-PS as well as TP2-C vs TP2-PS (Table S4). Bumblebee gut microbial communities did not differ between TPs and fertilization regimes in meso1. While the rhizosphere and endosphere experienced immediate effects of single fertilization events on microbial community composition even without a fertilization history, soil microbial community composition was only impacted by repeated fertilizer application, but not individual fertilization events. Flowers as well as earthworm associated microbial communities were impacted by short-term application of individual fertilization events without fertilization history, but not repeated fertilizer application.

3.3. Overlapping core microbiome across pig slurry fertilization history

The combined results from field and mesocosm experiments indicated that fertilization history determined the impact of PS fertilization on diversity and composition of microbiomes of soil and all plant compartments (Figure 2, 3) with individual fertilization events having only limited impact. Core members of a microbiome provide its functional stability across the trophic compartments and we therefore assessed to what extent fertilization history contributed to any changes in core members. Our analyses identified 458 unique core genera to be present with different relative abundances across all sampling TPs and treatments within the different compartments (Figure S4, shows genera with a minimum abundance of 5%; see Table S6 for details). The soil core microbiome only showed four identifiable genera, with *Bacillus* being shared with rhizosphere, endosphere and flowers in unfertilized field samples. The rhizosphere and endosphere core microbiomes overlapped in meso2 and field data in *Rhizobium, Pseudomonas* and *Novosphigobium* while *Steroidobacter* was present in endosphere irrespective of fertilization history (i.e., in all three data sets) (Figure 4). Core microbial community composition of flowers was very distinct for each of the data sets with overlaps between meso2 and field samples at the second sampling TP on PS sites in *Gaiellla, Microvirga* and *Mycobacterium* (Figure 4). The core microbiome of bumblebees was very consistent across the two

data sets and *Snodgrassella*, *Lactobacillus* and *Gilliamella* not only overlapped between meso1 and field samples, but also with the flower core microbiome within the second samplings of meso1, while there was no overlap between core genera of flowers and bumblebees in the field (Figure S4). Similarly, the earthworm core microbiome was consistent across meso1 and 2, with overlaps between the two data sets in *Flavobacterium, Luteolibacter* and *Aeromonas* as well as *Gaiella* overlapping with soil from meso2 (Figure S4). Taken together, the core microbiome diversities across all compartments largely overlapped between field and mesocosm samples and were hence relatively unaffected by the fertilization history. However, assessing the specific short-term impacts of PS fertilization events within the different fertilization histories requires the analysis of abundances of the different core taxa.

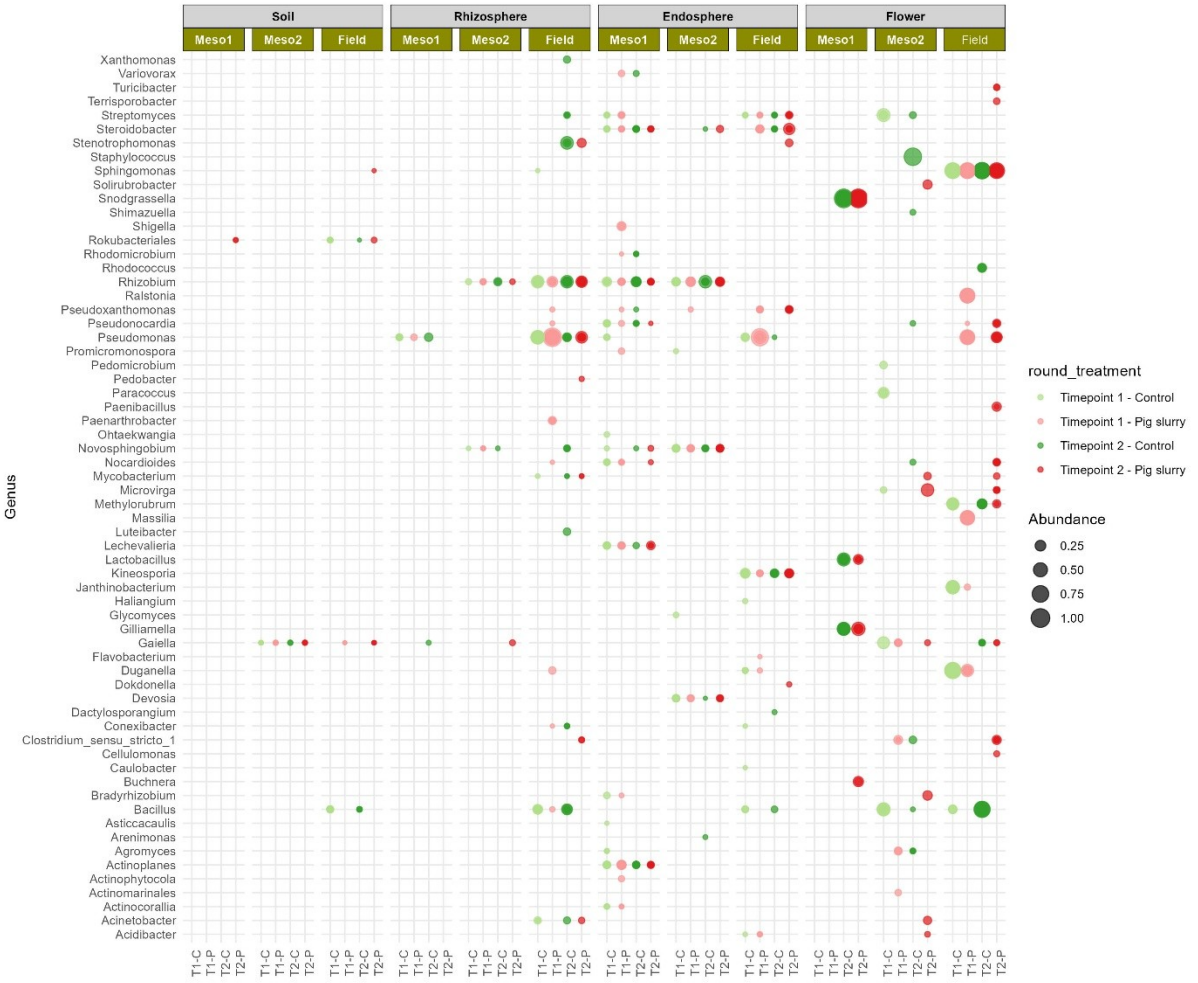

Figure 4: **Relative abundances within the core microbiome for the four most impacted trophic compartments.** The graph shows genera with a minimal relative abundance of 5% in all three datasets and trophic compartments, split by fertilization and sampling time point. Colours indicate sampling time point and fertilization, while the size of the dots represents the relative abundance of these genera.

3.4. Fertilization history determines effects of short-term application of pig slurry on differential abundances of core microbiota, but pig slurry associated taxa do not establish

As we did not detect major changes in core microbiome diversities between the three different data sets and hence their history of fertilization (Fig. 4), we next assessed the impact of individual applications of PS on the relative abundances of the different core taxa within each data set to test for potential short-term effects of PS fertilization on important bacterial genera. Differential abundance analyses using ANCOMBC2 on the core genera of all three data sets (meso1 and 2, field) resulted in 250 differentially abundant core genera in soil and plant compartments (Figure 5). Field samples showed the highest number of differentially abundant genera in all compartments. Flower samples only resulted in differentially abundant genera in the field data. Overall, TP1-PS and TP2-PS samples differed the most from TP1-C. There were only a few genera that were differentially abundant irrespective of fertilization history (i.e., in all three or at least in two datasets) across compartments and no taxon was differentially abundant irrespective of fertilization history (i.e., was present in all three datasets) within one compartment. Within field samples with a long-term history of fertilization, *Sphingomonas* were more abundant in PS-fertilized samples as compared to control samples in all plant compartments, including flowers, as well as in soil samples from meso2. Similarly, in both root compartments of long-term fertilization history field samples, *Mycobacterium* was differentially abundant in both directions upon pig slurry application. In the endosphere in meso1 and rhizosphere in meso2, *Mycobacterium* was significantly more abundant in samples collected at TP2 irrespective of fertilization. *Nocardioides* were more abundant in PS-fertilized samples in both root compartments in the field, while *Pirellula* was differentially abundant in soil samples of both mesocosm experiments with PS-fertilized samples having lower abundances in meso2. Additionally, in field soil samples *Gaiella* and *Pseudonocardia* were more abundant in PS-fertilized samples. These results showed that fertilization history affects the short-term effects of PS fertilization, i.e., within a long history of fertilization, a single PS application affected different important members of the core microbiome.

To assess whether the observed shifts in microbial composition and differences in abundance of core microbes are caused by an introduction of PS-associated microbial communities, we compared the differentially abundant core microbiome taxa to the microbial composition of the samples of the pig slurry applied to the field and mesocosm experiments. This comparison did not reveal PS-associated taxa to increase in abundance in any of the compartments. The composition of PS was dominated by *Clostridium_sensu_strico_1,* followed by *Terrisporobacter* in all three data sets (Figure S5). *Proteiniphilum* was the third most abundant genus in PS samples applied to the mesocosms while *Pseudomonas* was only found to be present with a relative abundance of at least 5% in samples of PS applied to the field. The latter was the only identifiable genus with a relative abundance of at least 5%

that was also identified as differentially abundant in the core microbiome. Even though being present in PS samples, *Pseudomonas* was less abundant in samples collected at TP2 irrespective of fertilization in rhizosphere samples from the field. These results therefore suggest that even after a long history of fertilization, PS-associated taxa are not becoming established and dominant in the different compartments.

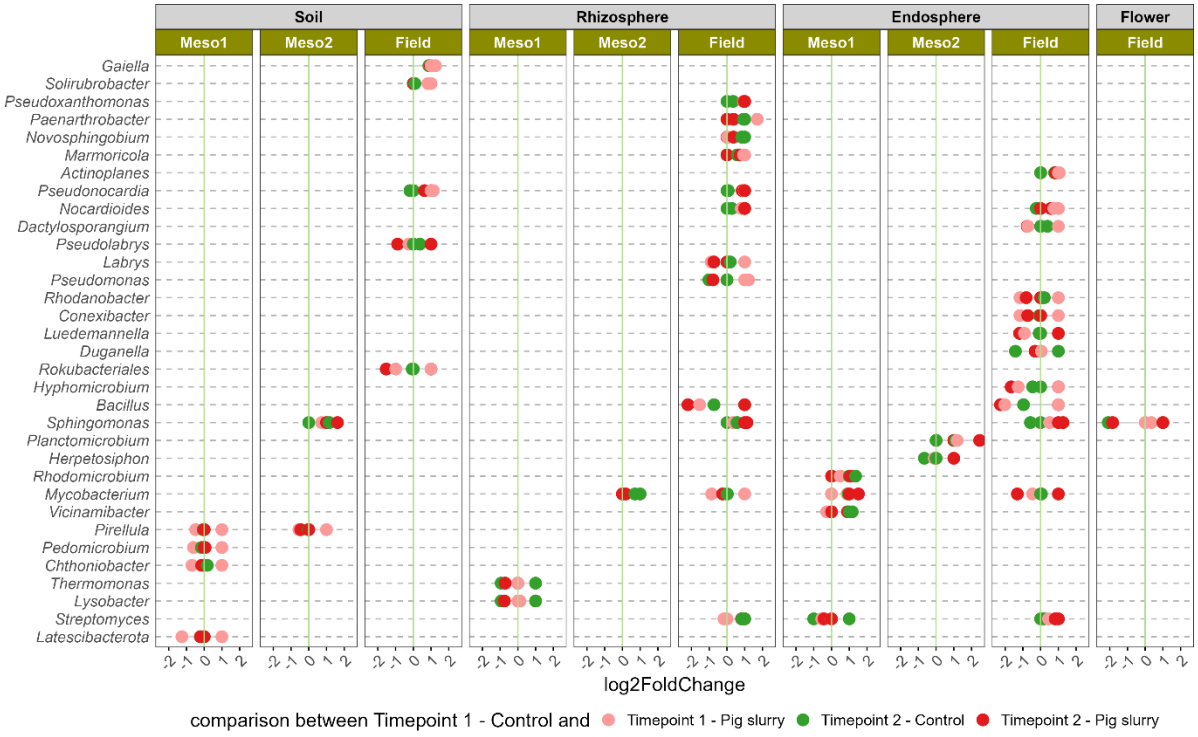

Figure 5: Differentially abundant core genera within soil, rhizosphere, endosphere and flower compartments in the field and both mesocosms. Differential abundances were calculated using ANCOM BC2 on the core genera. Bumblebees and earthworms did not yield significant results due to too few core genera in these compartments. Dots represent ASVs within a genus that are significantly more (positive values of log2FoldChange) or less (negative values of log2FoldChange) abundant in samples of pig slurry sites collected before or after fertilization, or from control sites collected at the second time point, compared to control sites sampled at the first time point.

3.5. Soil nutrients contribute to soil microbial composition

Given that the analysis of PS microbial composition did not suggest an establishment of PS-associated microbes in the different compartments, we assessed potential indirect effects of nutrient addition. The purpose of fertilization - adding nutrients to the soil to change nutrient availabilities for plants - might differentially support and reduce certain microbial taxa and thereby change microbial composition and abundances. Redundancy analyses of soil nutrient content resulting from the

electron-ultrafiltration of the field soil collected at both TPs and from both treatments revealed a strong impact of soil nutrient content on the bacterial composition. All nutrients together explained 56.1% of variation within the composition and with P, NO3 and Fe having the most significant effects. In the selected models, macronutrients (N, NO3, P, K, Ca, Mg, B, S) explained 40,10% (Figure S6A), while micronutrients (Fe, Na, Mn, Zn) explained 26,46% (Figure S6B). Comparing field control samples collected at TP1 to the respective samples from meso1 showed that, except for Ca, Na and S, which were higher in the meso1 soil, the nutrient content of the mesocosms was within or close to the range of the nutrient content of the field and hence comparable (Figure S6C). Overall, these analyses revealed strong impacts of soil nutrient content and indicate indirect effects on changes in the core microbial composition and abundances.

4. Discussion

In this study, we evaluated the effects of short-term and repeated application of PS as well as effects of a long-term history of fertilization on microbial communities across the trophic chain by setting up lab scale mesocosm experiments with previously unfertilized soil and sampling grassland fields with a long-standing fertilization routine. By replicating the grassland ecosystem in lab scale mesocosms, repeated PS applications in mesocosms aimed to reproduce grassland sites with a long-term history of PS fertilization. Sampling mesocosms and field sites after initial and repeated PS fertilization allowed to distinguish between short- and long-term effects of fertilizer application in grassland ecosystems in comparative microbiome analyses.

In contrast to previous work (Kraut-Cohen et al., 2023; Lazcano et al., 2013), our analyses revealed that soil microbial community composition changes under repeated, but not single, fertilizer applications, indicating legacy effects of a long fertilization history. The timing of sampling appears critical, as Suleiman et al. (2016) observed transient variations in soil microbial community structures following single PS application, with the community returning to its initial state after some time. Similarly, Tyrrell et al. (2023) found that manure-derived bacteria were no longer detectable in the soil after 10 weeks of manure application. Such transient shifts are likely based on indigenous microbiome members in respective compartments that outcompete introduced taxa after short-term application. However, supporting previous findings (Jetter et al., 2024; Mora-Salguero et al., 2025), our results indicate that the long-term history of fertilization with PS significantly impacts soil microbial communities by altering composition in field samples. In these field sites, even a short-term application of PS affected the soil microbial composition, indicating additive effects of long-term PS fertilization. Such fertilization legacy effects have been shown to still significantly alter soil microbial

community composition even 13 years after suspending fertilizer application (Zhang et al., 2018) and to be associated with the additional slow release of nutrients and dissolved carbon to all soil levels during manure decomposition (Diacono and Montemurro, 2010). The observed changes in our study may hence result not only from the establishment of fertilizer-associated taxa but also from indirect effects, such as changes in nutrient availability in soil with a long-term PS fertilization history (Jetter et al., 2024; Meier et al., 2021) as suggested by our differential abundance analysis in combination with the strong impacts of soil nutrient composition.

In contrast to soil microbial diversity, the alpha diversity of the root rhizosphere microbiome of *Trifolium pratense* decreased in field samples after an initial fertilizer application at the beginning of the year, but returned to the same level as control samples in samples taken after the second PS fertilization. Interestingly, a similar pattern (though not significant) was observed in meso2 but not meso1, suggesting that repeated PS fertilization had some effect on these transient microbial changes in the root compartments (Meier et al., 2021). Noteworthily, the first field sampling was conducted after the first PS fertilization in the field after winter, and meso2 was able to reproduce this effect. Previous studies have highlighted the impact of animal manure and fertilization on rhizosphere communities (Meier et al., 2021; Wu et al., 2023; Yu et al., 2024), which our data confirmed. In contrast to diversity, microbial community composition in the rhizosphere was already impacted by a single application of PS in meso1, in line with the effects seen in meso2 and in the field, indicating immediate effects of PS on the community composition of the rhizosphere irrespective of the fertilization history. Although rhizosphere and soil microbial communities are tightly interconnected, the observed short-term effect of fertilization on the rhizosphere in this study may be attributed to immediate changes in plant root exudates (Hao et al., 2022). Plants influence their rhizosphere bacterial communities (Turner et al., 2013) and respond to altered nutrient availability by selectively recruiting or rejecting bacteria, suggesting that fertilizer application enhances environmental filtering of microbial communities (Wu et al., 2023).

These rhizosphere effects can also extend to the endosphere. In our study, the composition of the endosphere microbiome changed following fertilization in both the mesocosm and long-term fertilization history field setups, consistent with previous findings (Ma et al., 2021). Based on non-significant differences between control samples and PS-fertilized samples at TP1 in meso1 and 2, our data revealed that short-term rhizosphere effects did not immediately translate into changes in the composition of the endosphere in meso1, but at TP2 in meso2. The effects are therefore slightly delayed reflecting an impact of fertilization history. This is expected, as root cell walls and the root immune system regulate access to the root interior (Tzipilevich et al., 2021). However, we found overlapping genera within the core microbial communities within the root compartments of field and

mesocosm plants. Notably, *Rhizobium*, *Pseudomonas*, and *Novosphigobium*, that comprise well-known root-associated plant-beneficial bacteria (Chouhan et al., 2021; Li et al., 2023; Molina et al., 2021; Santoyo et al., 2025), were core members in both the rhizosphere and endosphere in field and mesocosm samples, regardless of fertilization. Within these genera, various *Rhizobium sp*. (e.g. *Sinorhizobium ensifer*) can fix atmospheric nitrogen in legumes (Baxter et al., 2021), while *Rhizobium radiobacter* and *Pseudomonas fluorescence* strains were shown to enhance biotic and abiotic stress resilience in colonised plants (Sharma et al., 2008; Stringlis et al., 2018). In addition, these microbes have the ability to shape root microbiomes (Lagunas et al., 2023; Stringlis et al., 2018). The detection of these overlapping core genera within the root compartments of field and mesocosm plants underlines that lab-scale mesocosms can simulate effects of fertilization on the soil-root axis.

The observed short-term effects in roots were also found to influence the microbial composition of detritivores. While continuous fertilization effects on the earthworm gut microbiome have been previously documented (Bi et al., 2021), suggesting long-term fertilization legacy impacts similar to those seen in soil, the immediate changes in meso1 observed in *Lumbricus terrestris* in our study may be due to its feeding habits. As an anecic species*, L. terrestris* not only consumes soil but also plant material (Valckx et al., 2011). Therefore, changes in the microbial composition of the earthworm gut under short-term fertilization in our experiments can likely be attributed to shifts in the microbial communities of the rhizosphere and endosphere (Liu et al., 2018).

Aboveground, long-term PS application history in the field affected the microbial composition of flowers. This effect of fertilization history aligns with previous studies, which have reported a more homogeneous flower microbiome in long-term managed areas with more intensive fertilization (Gaube et al., 2020). While the phyllosphere, including flower microbial communities, has been shown to filter microbes originating from the soil to some extent (Massoni et al., 2021), our study found that the flower microbial communities were still susceptible to compositional changes under long-term fertilization. Additionally, short-term effects of PS on flower microbial composition were observed in the first mesocosm experiment. Interestingly, these changes occurred even when fertilizer was applied to mown boxes that lacked floral tissues. Fertilization may alter the composition of leaf microbiomes (Gaube et al., 2020), and phyllosphere community members might be transferred to emerging flowers, thereby influencing their composition. Consistent with this, large proportions of flower microbiomes originate from soil or the leaf phyllosphere microbiome (Massoni et al., 2021), and certain Streptomyces strains can reach flowers via vascular bundles (Kim et al., 2019). As a result, flowers serve as reservoirs for microbes—both beneficial and potentially pathogenic—that can be transmitted to pollinators (Keller et al., 2021; Vannette, 2020) and spread throughout the environment.

Flower core microbiomes were very distinct depending on the fertilization history although some overlapped between mesocosm experiments and field samples. Flower-specific *Microvirga* (Ottesen et al., 2013), for instance, was detected in meso2 and in the field, both depicting a certain history of fertilization. *Snodgrassella, Lactobacillus* and *Gilliamella*, core gut microbiome members of honeybees and bumblebees (Kwong et al., 2017; Zhang et al., 2021), overlapped with the bumblebee core microbiome within meso1. These results suggest an interaction of bumblebees and flowers in our indoor meso1 set up. Such overlaps were not detected in our field data. However, in the field, it is to be expected that sampled bumblebees might have also foraged on sites with a history of no or less intensive fertilization, resulting in higher flower diversity and hence resource availability (Birkenbach et al., 2024). While adding more complexity and a longer history of fertilization to mesocosm designs might be necessary to fully rebuild long-term history effects from the field, these results indicate that our mesocosms can at least partially simulate critical ecosystem interactions that are essential to understand the intricate interplay of microbial members of the leaf-flower-insect axis.

Changes in the microbial composition of the different compartments after PS fertilization were driven by important native members of the core microbiome differing in their abundances. Especially a long-term history of fertilization with PS in the field caused genera associated with important ecosystem functions to change in abundance. In flowers, *Sphingomonas,* an important player in pathogen defense in the phyllosphere microbiome (Innerebner et al., 2011), decreased after repeated fertilization. In contrast, in belowground samples, i.e., soil and roots, *Gaiella, Pseudonocardia, Sphingomonas* and *Nocardioides* were more abundant in long-term PS-treated samples. Species within these genera are involved in remediation of polluted soils and degradation of environmental contaminants (Gatheru Waigi et al., 2017; Jiao et al., 2019; Riahi et al., 2022) as well as in phytoremediation and modulating endophytic microbial interactions (Gatheru Waigi et al., 2017; Li et al., 2021; Wang et al., 2022) and have been shown to change in abundance in response to altered soil nitrogen levels (Meier et al., 2021). The higher abundances of these genera in flower and root compartments might illustrate the disturbing legacy impact of long-term organic fertilization history on microbial communities and a readjustment of plant stress adaptive processes (Spooren et al., 2025).

Introduced PS-associated and hence allochthonous taxa did not significantly increase in abundance in the core microbial communities of the different compartments and did therefore not fully establish after short-, but also not under long-term PS application. However, risk factors like antimicrobial resistance genes originating from animal manure might still be transferred to the indigenous, more adapted, soil microbial community via horizontal gene transfer and accumulate in the soil (Dimitriu, 2022; Thomas and Nielsen, 2005; Udikovic-Kolic et al., 2014). Transient changes in the soil microbial

communities due to organic fertilization may still entail legacy effects of land use on soil microbial communities and even translate to higher trophic compartments (Liu et al., 2019). Assessing microbial communities across the trophic chain and focusing on both short- and long-term effects including different fertilization histories and associated legacy effects of fertilization will be necessary to establish sustainable land use in a well-balanced ecosystem.

5. Conclusions

Assessing both mesocosms and field samples revealed that different histories of PS fertilization alter the bacterial composition of key compartments across the trophic chain in grassland ecosystems and their effects towards a repeated application of fertilizer, with significant effects on belowground compartments. Effects of short-term application were most pronounced in root compartments, where *Trifolium pratense* immediately responded to altered nutrient availability even after the first application of PS by changing microbial community composition in the rhizosphere. In contrast, soil and flower microbial communities were more influenced by long-term fertilization history in the field, than by single fertilization events without a fertilization history. By replicating field conditions, our study demonstrates that lab-scale mesocosms provide a reliable system to recreate grassland ecosystems with trophic interactions. Repeated application of fertilization in meso2 caused similar effects as those observed in field samples with a long-term history of PS fertilization in some compartments, and a continuation of fertilizer application might fully recreate effects in other compartments as well, enabling the analysis of both short-term fertilizer application and legacy effects of repeated fertilization. Following the eco-holobiont concept (Singh et al., 2020) a holistic and complex mesocosm approach including multiple compartments across the trophic chain is essential for assessing the intricate interplay and effects of anthropogenic activities. This study highlights the previously unexplored effects of a history of long-term fertilization on the short-term effects of a single fertilizer application across several compartments, paving the way for further complex mesocosm studies and contributing to a more holistic understanding of eco-holobiont systems.

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7. Data availability

The datasets generated during and/or analyzed during the current study conducted in cooperation with the Biodiversity Exploratories program (DFG Priority Program 1374) are available in the bexis data base (DatasetIDs: dataset IDs 32176, 32177, 31937) as well as publicly available on GitHub (https://github.com/kunaljaani/Fertilization-impacts-microbiomes-along-the-grassland-trophic-chain/tree/9ed11ecadad8fd6b491dbffe77abdcfd9ff1747e/nutrientData) and on NCBI (Bioproject number: PRJNA1188490).

8. Conflict of interests

The authors declare no conflicting interests.

9. Authors contributions

Conceptualization: S.S., L.W., C.R. and P.S.; Investigation: Ka.J., Ku.J., K.W., U.S. and R.C.; Formal Analysis: Ka.J.; Data Curation: Ka.J., Ku.J., S.S., L.W., C.R. and P.S.; Writing – Original Draft: Ka.J.; Writing – Review & Editing: Ka.J., Ku.J., S.S., L.W., C.R. and P.S.; Funding Acquisition: S.S., L.W., C.R. and P.S.; Supervision: S.S., L.W., C.R. and P.S.

10. Abbreviations

PS: pig slurry

C: control

TP: sampling time point

meso1: first mesocosm experiment

meso2: second mesocosm experiment

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Supplemental Material

Effects of Pig Slurry Fertilization History on Grassland Microbiomes: Insights from Field and Mesocosm Experiments

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Experimental set up of mesocosm 1

Mesocosm experiments were setup in a laboratory at the University of Ulm. Room temperature was set to 22°C with a light phase of 16hours. The first setup was built up out of eight 40x60x15cm euroboxes (40x60x15cm) with drainage holes each in an individual flight cage (60x60x80cm). The top soil layer (up to 15cm depth) of an unfertilized site in the Botanical Garden at the University of Ulm was collected for the experimental setup. *Trifolium pratense* (red clover) seeds (Samen Fetzer, Reutlingen-Gönningen, Germany) were germinated in petri dishes and transplanted to the boxes at the two-leaf stage. Each box was equipped with 15cm of soil and eight clover plants as well as a community of common grasses (*Festuca rubra, Lolium perenne, Poa pratensis*) which were sown into the box at time of transplant of the clover seedlings. Four out of eight boxes were fertilized with 250ml of pig slurry (diluted to 500 ml with water) at the beginning of the setup, the other four boxes were

left unfertilized. Pig slurry was applied by mixing liquid and solid parts and equally pouring the slurry onto the mown plant cover using a beaker. As soon as each clover plant of a box was flowering a microcolony of 32 bumblebee workers (*Bombus terrestris*) was introduced to the setup within the flight cage to forage and pollinate the plants. One week after the introduction of bumblebees, all bumblebees and four plants of each box were sampled. The remaining clover plants and the grass of all boxes were cut off at about 3cm height to imitate mowing and the four pig slurry-fertilized boxes received another fertilization treatment similar to the first one (with 250ml diluted to 500 ml with water). After re-flowering of the clover pants new microcolonies were introduced into the flight cages and the remaining four clover plants and all bumblebees were sampled one week after introduction of bumblebees.

Four Bumblebee (*Bombus terrestris*) mother colonies were ordered from Biobest (https://www.biobestgroup.com/de). Microcolonies of bumblebees consisted of 32 age standardized workers, eight workers originating from each of the four mother colonies. Bumblebees were taken out of the mother colony latest two days after emerging and were left in the microcolony for a week before introduction into the flight cages to acclimatize and acquire a common microbiome. During the whole course of the experiment (in acclimatization phase and when being in the flight cage) all bumblebees were fed additionally with sugar water solution to guarantee survival.

Experimental set up of mesocosm 2

After the first mesocosm experiment, big plant parts, especially roots from grasses, were removed from the soil. The soil from boxes of the respective treatments was homogenized and reused to fill the pots for the second mesocosm experiment. Here, 20 clover plants were individually sown into 13x13x15cm pots in the same laboratory under the same conditions. 10 plants grew in the soil from pig slurry fertilized boxes, 10 grew in soil from control boxes. As soon as the plants started flowering, pig slurry treated plants were fertilized with 20ml of pig slurry. Pig slurry was applied by diluting the slurry with 30 ml of water to make application easier and poured onto the soil with a beaker. 33 days

after flowering half of the plants were sampled, while the other half was cut off at about 3 cm height to simulate mowing, followed by another fertilization in the same way as the first. 33 days after reflowering those plants were samples as well.

Pig slurry used in the mesocosms was collected from the local farmers on the Swabian alb. Pig slurry was stored at 4°C and was tested for vital bacteria by plating before the use in the experiment (data not shown). The amount of pig slurry applied to the individual boxes was deduced from information received by the local pig slurry farmers on the Swabian Alb. Amounts were calculated down to the size of the boxes and pots and are equal to a fertilization of $10m^3$/ha.

All plants in both experimental set ups were watered ad libitum.

Bioinformatic processing

Bioinformatic processing was conducted in QIIME2 (Evan Bolyen et al., 2019) using the DADA2 pipeline as previously described in (Jetter et al., 2024) The three datasets were processed individually, while for the field samples, the denoising steps of the two runs were conducted separately and the files of representative sequences, metadata and the dada2 output table were merged before taxonomical assignment and further processed as one set of sequences. For the field samples reads were trimmed at 30bp and 240bp for forward and 30bp and 215bp/220bp for reverse reads, for the samples of the first mesocosm experiment at 50bp and 200bp forward and reverse reads and for the samples of the second mesocosm experiment at 30bp and 250bp forward and 30bp and 230bp reverse reads. Further bioinformatic processing was conducted as previously described in (Jetter et al., 2024) separately for the three data sets. In short, Taxonomy was assigned using q2-feature classifier-sklearn (version 0.24.1) (Bokulich et al., 2018) in Qiime2 (Evan Bolyen et al., 2019) with the SILVA 138-99 database (Quast et al., 2013), phylogenetic trees were build using fasttree (Price et al., 2010) after aligning sequences with mafft (Katoh et al., 2002) and trees were rerooted in dendroscope (Huson et al., 2007) using an uncultured archaeal sequence (accession number KT433146.1). All three datasets, including the phylogenetic trees, the meta data and created biom tables were merged into three

different phyloseq objects in the R programming language (R Core Team, 2025) using the packages phyloseq (McMurdie & Holmes, 2013) and microbiome (Lathi & Shetty, 2019) to conduct further analyses.

Supplementary Tables

TableS1: Sample sizes for field and mesocosm samples collected at two different time points from five different trophic compartments and two different treatments. C represents control samples, PS samples fertilized with pig slurry. First and second indicate the different sampling time points.

		Soil		Rhizosphere		Endosphere		Earthworm		Flower		Bumblebee		Slurry	Total
		C	PS	C	PS	C	PS	C	PS	C	PS	C	PS	PS	
Field	T1	24	24	24	24	24	24	-	-	24	24	18	4	4	218
	T2	24	16	24	16	24	16	-	-	24	16	19	9	-	188
Meso1	T1	3	2	15	13	15	14	8	2	8	7	5	4	1	97
	T2	4	4	15	11	15	11	6	3	15	11	5	5	-	105
Meso2	T1	5	5	5	5	5	5	4	4	5	5	-	-	1	49
	T2	5	5	5	5	5	5	5	3	5	5	-	-	-	48
Total		65	56	88	74	88	75	23	12	81	68	47	22	6	705

Table S2: Results of linear mixed models followed by emmeans pairwise comparisons of samples of field, Mesocosm1 and mesocosm 2 using holm p-value adjustment. Significant values are marked in **bold**.

Comparison		TP1-C - TP1-PS	TP1-C - TP2-C	TP1-C – TP2-PS	TP1-PS – TP2-C	TP1-PS – TP2-PS	TP2-C-TP2-PS
Soil	Meso1	0.483	0.759	0.726	0.316	0.726	0.571
	Meso2	1.000	1.000	1.000	1.000	1.000	1.000
	Field	0.980	**0.017**	0.747	1.00	0.98	1.00
Rhizosphere	Meso1	0.842	1.000	1.000	0.756	0.086	1.000
	Meso2	1.000	1.000	1.000	1.000	1.000	1.000
	Field	**0.008**	1.000	1.000	**0.003**	**0.015**	1.000
Endosphere	Meso1	0.361	0.103	0.264	**0.026**	**0.009**	0.881
	Meso2	1.000	1.000	1.000	1.000	1.000	1.000
	Field	0.857	0.998	0.467	0.425	0.998	0.199
Flower	Meso1	1.000	0.466	1.000	1.000	1.000	1.000
	Meso2	0.180	0.951	0.951	0.180	0.951	0.951
	Field	0.673	**0.001**	0.324	0.456	0.673	0.673
Earthworm	Meso1	1.000	0.181	1.000	1.000	0.343	1.000
	Meso2	1.000	0.533	0.907	0.907	0.943	1.000
Bumblebee	Meso1	1.000	1.000	1.000	1.000	1.000	1.000
	Field	1.000	1.000	1.000	1.000	1.000	1.000

Table S3: Results of PERMANOVA and pairwise PERMANOVA of field samples using Bray-Curtis distances. Significant values are marked in **bold**.

Comparison	Soil	Rhizo-sphere	Endo-sphere	Flower	Bee
Overall F	**0.001** (R^2=0.126)	**0.001** (R^2=0.078)	**0.001** (R^2=0.094	**0.001** (R^2=0.046)	0.060 (R^2=0.017)
Overall TP	**0.032** (R^2=0.021)	**0.001** (R^2=0.040)	**0.001** (R^2=0.021)	**0.001** (R^2=0.080	**0.029** (R^2=0.036)
Overall TP*F	0.177 (R^2=0.010)	**0.001** (R^2=0.029)	**0.048** (R^2=0.011)	**0.001** (R^2=0.026)	0.481 (R^2=0.021)
TP1-C - TP1-P	0.18 (R^2=0.157)	**0.001** (R^2=0.122)	**0.004** (R^2=0.108)	**0.001** (R^2=0.106)	
TP1-C - TP2-C	**0.001** (R^2=0.039)	**0.001** (R^2=0.047)	**0.010** (R^2=0.029)	**0.001** (R^2=0.105)	
TP1-C – TP2-P	**0.013** (R^2=0.146)	**0.001** (R^2=0.124)	**0.003** (R^2=0.116)	**0.010** (R^2=0.116)	
TP1-P – TP2-C	**0.001** (R^2=0.146)	**0.001** (R^2=0.122)	**0.025** (R^2=0.114)	**0.001** (R^2=0.135)	
TP1-P – TP2-P	0.111 (R^2=0.032)	**0.001** (R^2=0.107)	**0.001** (R^2=0.044)	**0.001** (R^2=0.119)	
TP2-C-TP2-P	**0.001** (R^2=0.116)	**0.001** (R^2=0.096)	**0.001** (R^2=0.105)	**0.325** (R^2=0.050)	

Table S4: Results of PERMANOVA and pairwise PERMANOVA of Mesocosm1 samples using Bray-Curtis distances. Significant values are marked in **bold**.

Comparison	Soil	Rhizo-sphere	Endo-sphere	Earthworm	Flower	Bee
Overall F	0.313 (R^2=0.090)	**0.001** (R^2=0.034)	**0.001** (R^2=0.028)	**0.002** (R^2=0.094)	**0.004** (R^2=0.044)	0.675 (R^2=0.088)
Overall TP	0.813 (R^2=0.076)	**0.001** (R^2=0.045)	**0.001** (R^2=0.079)	**0.002** (R^2=0.216)	**0.018** (R^2=0.051)	0.755 (R^2=0.039)
Overall TP*F	0.656 (R^2=0.084)	**0.014** (R^2=0.023)	0.360 (R^2=0.016)	0.418 (R^2=0.039)	0.314 (R^2=0.025)	0.763 (R^2=0.028)
TP1-C - TP1-P		**0.002** (R^2=0.052)	0.338 (R^2=0.079)	0.872 (R^2=0.075)	0.437 (R^2=0.073)	
TP1-C - TP2-C		**0.001** (R^2=0.069)	**0.001** (R^2=0.140)	**0.002** (R^2=0.205)	0.060 (R^2=0.072)	
TP1-C – TP2-P		**0.001** (R^2=0.064)	0.278 (R^2=0.106)	**0.002** (R^2=0.410)	0.899 (R^2=0.036)	
TP1-P – TP2-C		0.058 (R^2=0.092)	**0.001** (R^2=0.183)	0.230 (R^2=0.170)	**0.002** (R^2=0.143)	
TP1-P – TP2-P		**0.004** (R^2=0.071)	**0.001** (R^2=0.147)	0.075 (R^2=0.519)	0.063 (R^2=0.084)	
TP2-C-TP2-P		**0.002** (R^2=0.067)	**0.001** (R^2=0.130)	**0.045** (R^2=0.190)	0.051 (R^2=0.067)	

Table S5: Results of PERMANOVA and pairwise PERMANOVA of Mesocosm2 samples using Bray-Curtis distances. Significant values are marked in **bold**.

Comparison	Soil	Rhizo-sphere	Endo-sphere	Earthworm	Flower
Overall F	**0.010** (R^2=0.070)	**0.001** (R^2=0.104)	**0.013** (R^2=0.080)	0.690 (R^2=0.040)	0.094 (R^2=0.057)
Overall TP	0.068 (R^2=0.060)	**0.003** (R^2=0.090)	**0.00**3 (R^2=0.098)	0.051 (R^2=0.137)	0.581 (R^2=0.050)
Overall TP*F	0.404 (R^2=0.178)	0.111 (R^2=0.055)	0.127 (R^2=0.061)	0.770 (R^2=0.037)	0.140 (R^2=0.055)
TP1-C - TP1-P		**0.0.031** (R^2=0.163)	0.169 (R^2=0.135)		
TP1-C - TP2-C		0.075 (R^2=0.136)	**0.046** (R^2=0.159)		
TP1-C – TP2-P		**0.001** (R^2=0.195)	**0.015** (R^2=0.187)		
TP1-P – TP2-C		**0.001** (R^2=0.216)	**0.003** (R^2=0.193)		
TP1-P – TP2-P		**0.004** (R^2=0.183)	**0.010** (R^2=0.187)		
TP2-C-TP2-P		**0.001** (R^2=0.190)	**0.001** (R^2=0.181)		

Table S6: Number of core genera found within the different compartments and respective treatment–sampling time point combinations and data sets.

Data	Fertilization	Time point	Soil	Rhizosphere	Endosphere	Earthworm	Flower	Bumblebee
Field	C	1	98	114	130	-	10	1
Field	PS	1	104	73	148	-	11	0
Field	C	2	114	102	119	-	21	2
Field	PS	2	91	137	121	-	25	2
Meso1	C	1	182	204	88	111	1	4
Meso1	PS	1	140	180	71	130	1	3
Meso1	C	2	160	188	172	115	4	4
Meso1	PS	2	156	196	163	118	5	6
Meso2	C	1	148	197	114	75	17	-
Meso2	PS	1	141	173	145	91	71	-
Meso2	C	2	156	180	148	119	44	-
Meso2	PS	2	151	168	137	127	53	-
unique			266	355	310	186	112	9

Supplementary Figures

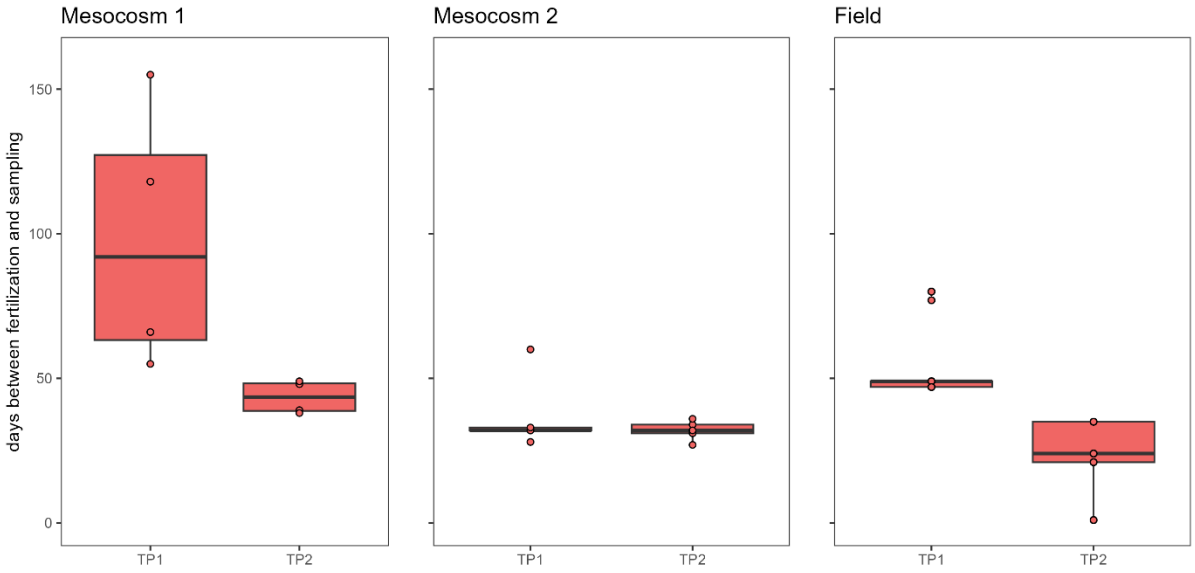

Figure S1: Days between fertilization and sampling for the two samplings (TP1 and TP2) of pig slurry fertilized samples of the three different sets of samples. Days were counted from the day of the last fertilization to the day of sampling. Boxes indicate the time for the two different sampling time points. All set ups were fertilized in the beginning, followed by the first sampling at TP1, another fertilization and sampling at TP2.

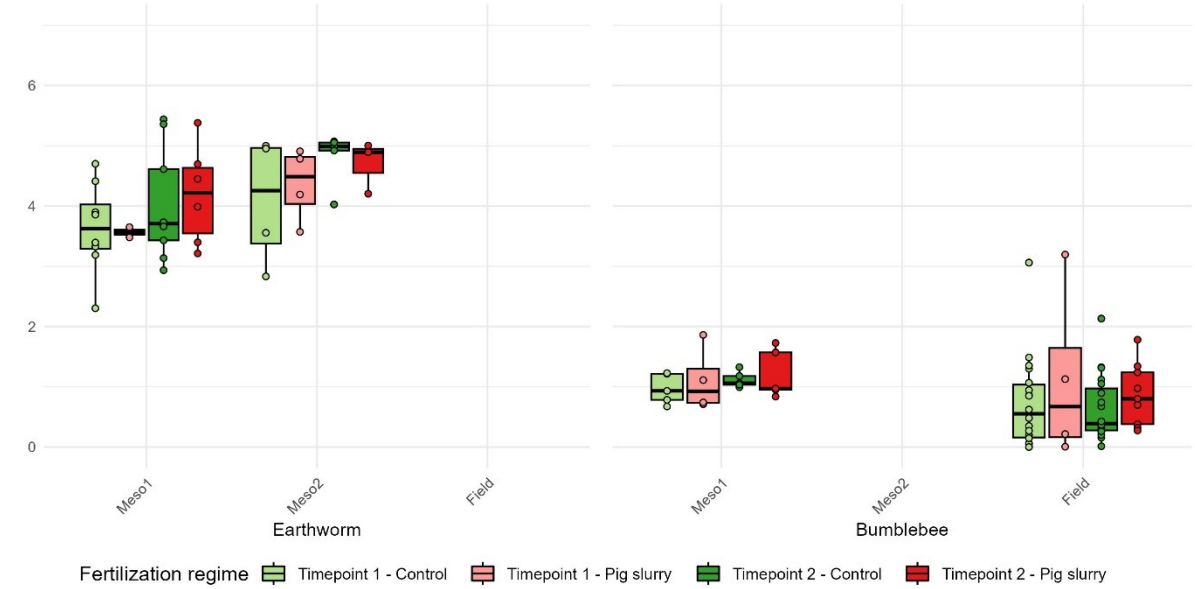

Figure S2: Alpha diversity of earthworm and bumblebee samples. Bars are separated by the different set of samples. Shannon index was calculated for every dataset and compartment individually. Colours indicate control or pig slurry fertilized samples as well as the sampling round. Differences between samples were tested using a linear model followed by emmeans post hoc tests, but differences were all not significant.

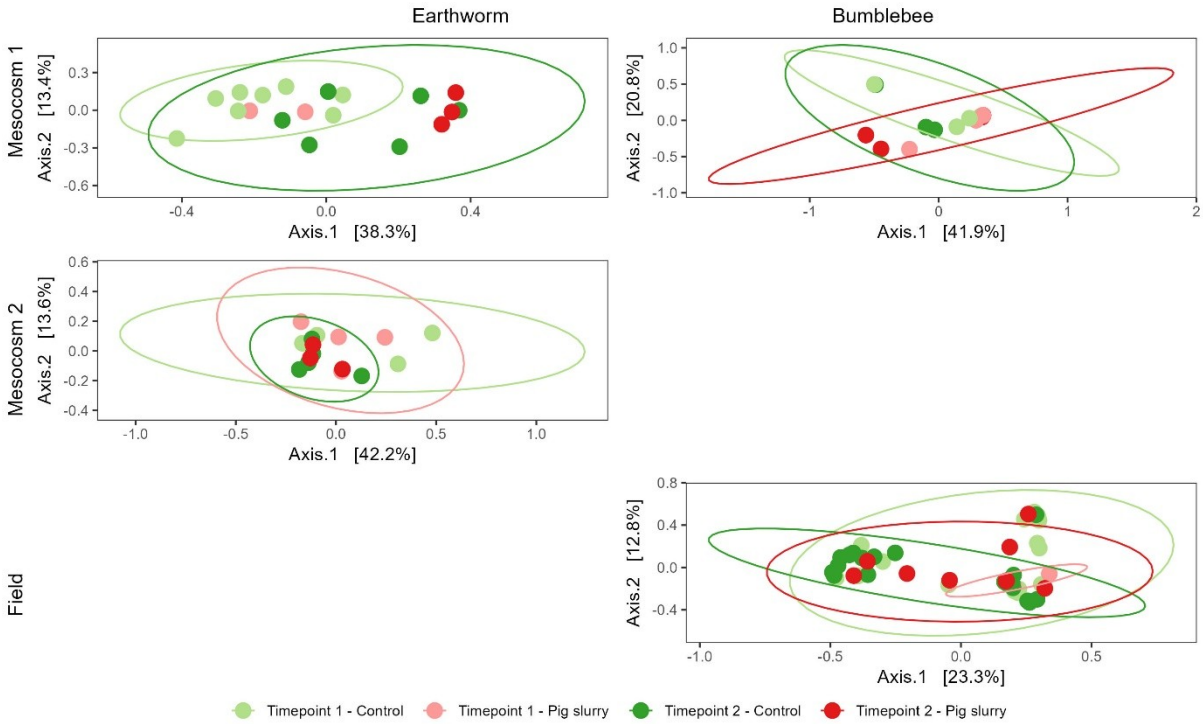

Figure S3: Beta diversity based on Bray-Curtis-distances in multidimensional scaling. Earthworm and bumblebee samples are shown for the three different datasets. Colors indicate control or pig slurry fertilized samples as well as the sampling round.

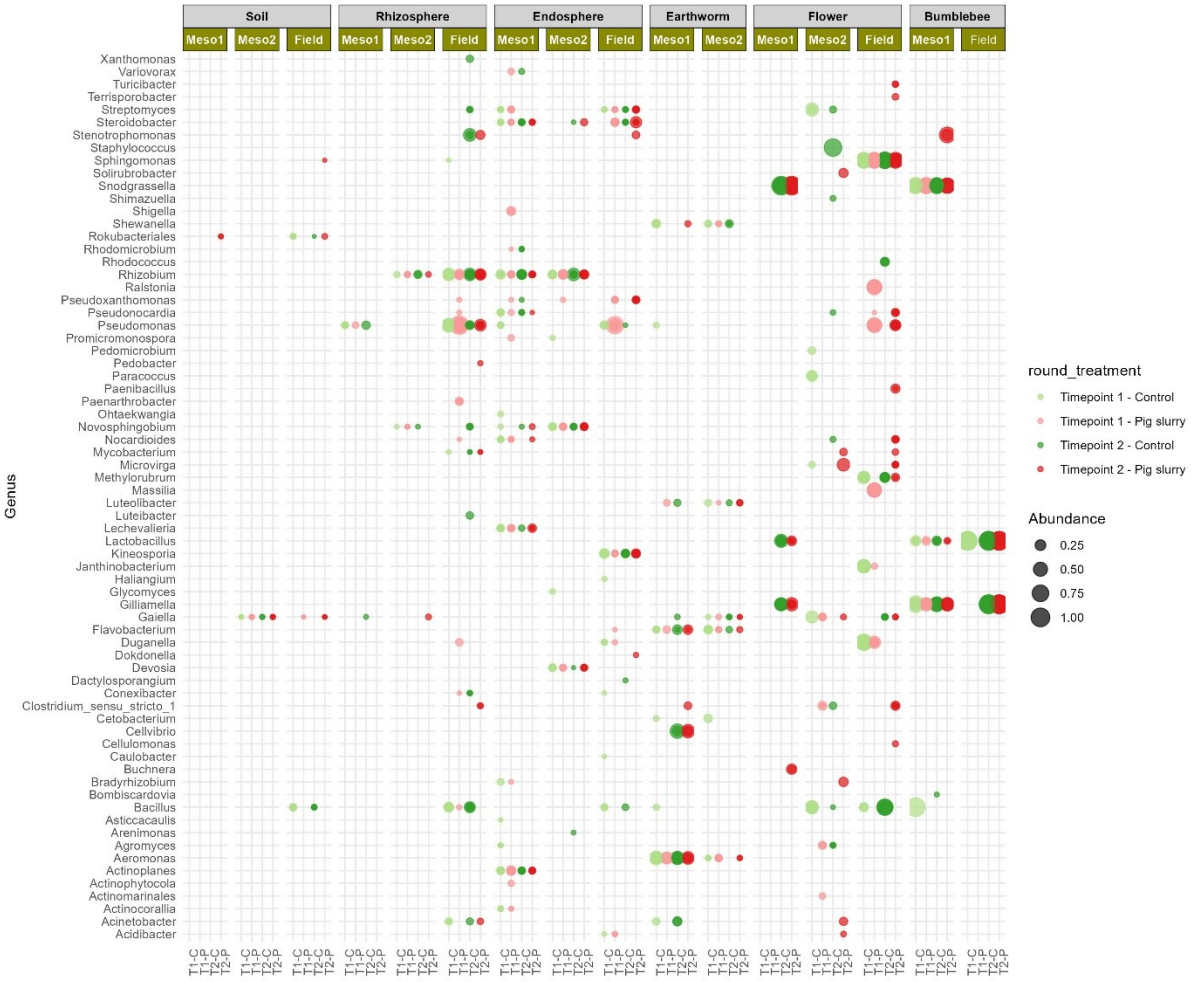

Figure S4: Relative abundances within the core microbiome of all trophic compartments. Graph shows genera with a minimal relative abundance of 5% in all three datasets and trophic compartments split by fertilization and sampling time point. Colours indicate sampling time point and fertilization while the size of the dots represents the relative abundance of these genera.

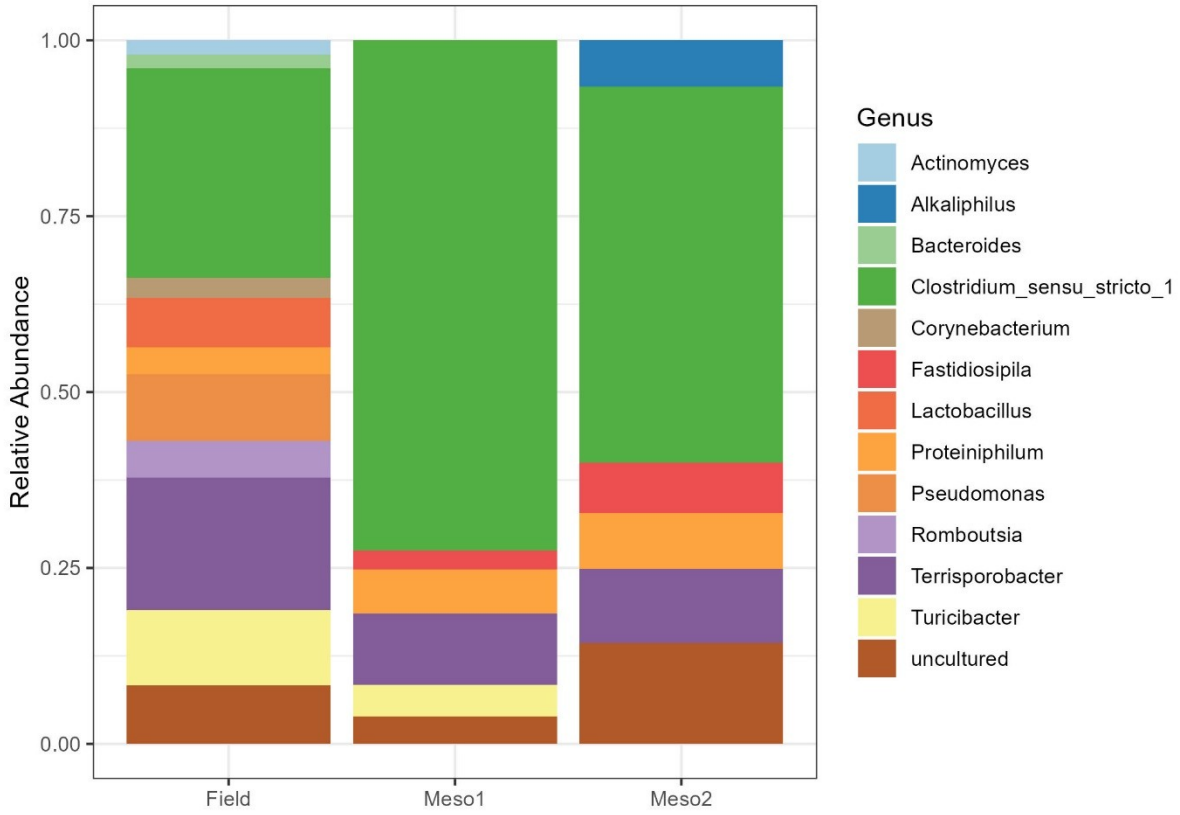

Figure S5: Composition of pig slurry. Composition shows genera with a relative abundance of at least 5%. For field and mesocosm1, the bar represents a pool of four samples, for mesocosm2 one individual sample.

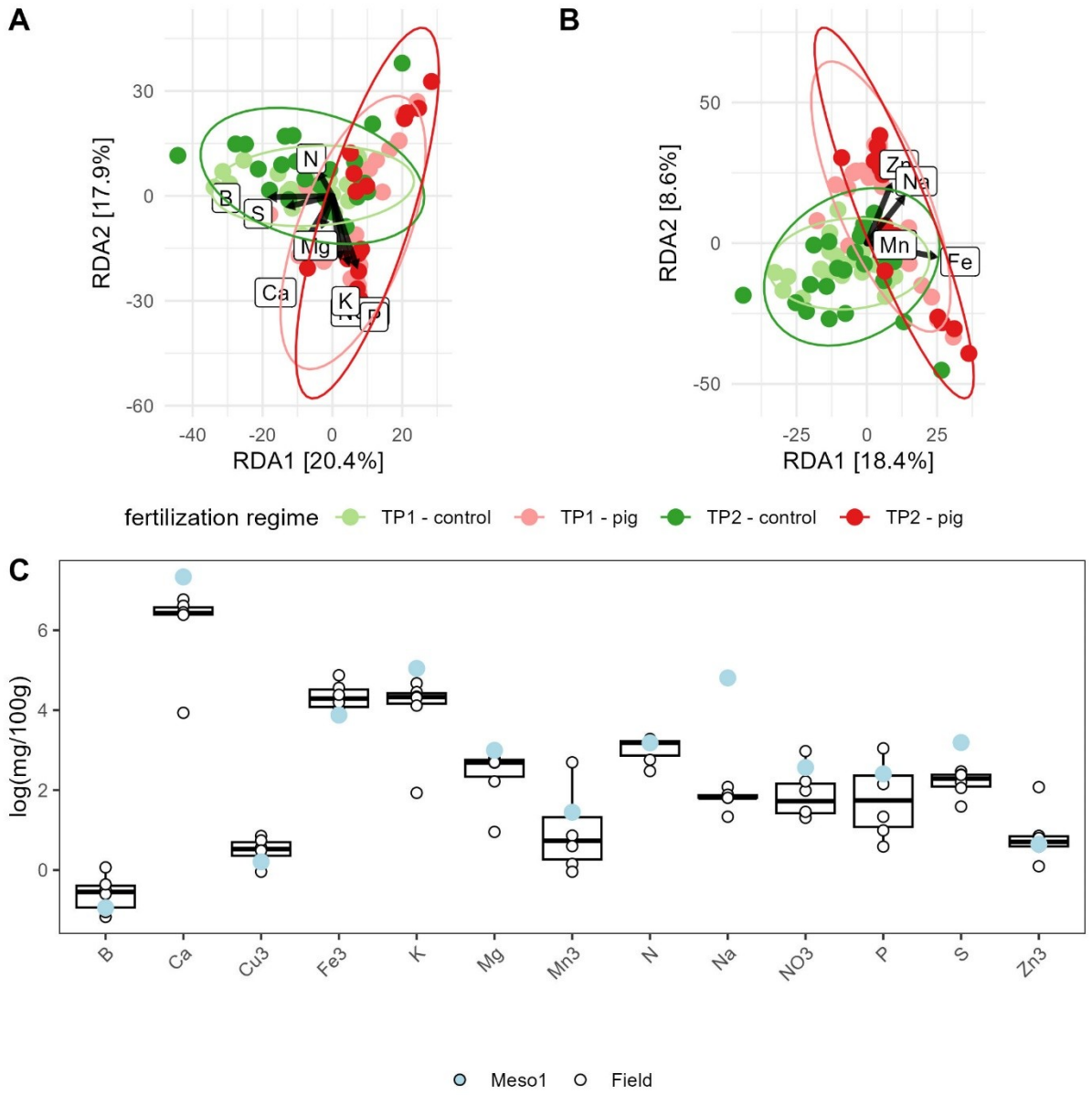

Figure S6: Soil nutrient content. RDA analysis of Macro (A) and Micro (B) nutrients contributing to the soil microbial communities in the field samples collected at two different time points on control sites (green) or on pig slurry fertilized sites (red). Range of soil nutrient content (C) in macro and micronutrients in field (white) and mesocosm1 (blue) samples, both collected on control sites at TP1.

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Statement of contribution

I am first author on all publications and manuscripts included in this dissertation. The detailed contribution to every chapter is listed below.

1. Advances in understanding soil microbiomes in ecosystem functions across trophic chains

Authors: Karoline Jetter, Patrick Schäfer

Book chapter in Dunfield, K. (ed.), Understanding and utilising soil microbiomes for a more sustainable agriculture, Burleigh Dodds Science Publishing, Cambridge, UK

This review was published as book chapter in the book *Understanding and utilising soil microbiomes for a more sustainable agriculture* in Burleigh Dodds Science Publishing. I am first author of this book chapter and conducted the literature review and wrote the first draft of the chapter. Patrick Schäfer and me worked on the revisions for the final version.

2. Fertilization impacts microbiomes along the grassland trophic chain

Authors: Karoline Jetter, Kunal Jani, Kerstin Wilhelm, Ulrike Stehle, Rostand R.Chamedjeu, Christian U. Riedel, Lena Wilfert, Patrick Schäfer, Simone Sommer

Journal: ISME communications

This manuscript was published as peer-reviewed article in the journal ISME communications. Kunal Jani and me are shared first authors. Together with bachelor students and Rostand Chamedjeu, I conducted the fieldwork and sampling and substantially contributed to sample processing and lab work. I conducted the bioinformatic processing as well as the first half of the statistical analysis and created the figures. I wrote the first draft of introduction, material and methods, as well as results and all co-authors substantially contributed to the revisions of the editor and reviewers.

3. Effects of Pig Slurry Fertilization History on Grassland Microbiomes: Insights from Field and Mesocosm Experiments

Authors: Karoline Jetter, Kunal Jani, Kerstin Wilhelm, Ulrike Stehle, Rostand R.Chamedjeu, Christian U. Riedel, Simone Sommer, Lena Wilfert, Patrick Schäfer

Journal: Science of the Total Environment

This manuscript was submitted to the peer-review journal Science of the Total Environment and was published after the defense of this thesis. I am first author and established the mesocosm experiments. I conducted the field work with bachelor students and Rostand Chamedjeu and the sampling of the mesocosms as well as the processing of the samples. I conducted all bioinformatic processing as well as the statistical analysis, visualized the results and wrote the first draft of the manuscript.

Eidesstattliche Versicherung

Hiermit versichere ich, Karoline Jetter, dass ich die Arbeit

„Composition of microbiomes along the trophic chain in agricultural grassland ecosystems"

selbstständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, sowie die wörtlich oder inhaltlich übernommenen Stellen als solche kenntlich gemacht habe und diese im Literaturverzeichnis aufgeführt sind. Large language models (chatGPT, DeepL write) wurden nur zur Überprüfung der Grammatik sowie zur Verfeinerung einzelner Sätze verwendet.

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Karoline Jetter

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Curriculum Vitae

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