

Soil Biotic Complexity Shapes Grassland Bacterial Communities and Ecosystem Functioning

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Summary for general public

Soils are everywhere and they are teeming with life. They contain over 59% of all biodiversity and these different species are in close contact with each other. Soil organisms interact with such a vast array of other soil organisms that they construct an intricate soil community network, which is still poorly understood. However, global change is pushing down on these smaller life forms and, by extent, threatening the world as we know it. The loss in soil biodiversity is tangible in the functional network and stunts the performance of entire ecosystems.

This thesis will point our gaze downwards, literally. We will explore the intricate soil network, comprised of life such as microscopic insects, worm-like organisms, fungi and bacteria. While largely invisible, the cooperation of these organisms keeps both the natural world and our civilisation running, as they deliver valuable ecosystem functions which shape our aboveground worlds. Bacteria are an essential cornerstone in soil networks. For instance, they are crucial in the decomposition process, in which they recycle organic matter and thus revitalise the ecosystem. This was our reason to further look into how Bacteria are impacted by the loss of whole groups from the soil network.

This research has found that subsequently losing more and more different organism groups results in bacterial communities which differ significantly from each other. As their composition changes, the benefits they provide to the ecosystem change as well. We showed that the ability of bacteria to recycle nutrients is drastically impeded as the soil networks are degrading. This will in turn cause further decline in biodiversity as certain species will be unable to sustain their nutritional needs

Plant productivity was affected as well. While grasses seemed to be more resilient, herbs decreased in their performance, which could ultimately have important consequences for the ecosystems, such as a lower abundance of flowers for pollinators.

Summary for scientific peers

Soil organisms sustain the majority of all terrestrial life. They are indispensable as they perform ecosystem functions which are often taken for granted, even by scientists. Besides their high biodiversity, soil communities are also made up of a wide variety of taxonomic groups, especially those small in size: Mesofauna, Microfauna, Fungi, Protists, Bacteria and Archaea. This complexity in the soil – and the interactions between the different agents – is the driver of ecosystem multifunctionality and the stabilisation of ecosystems. With global change, many chackles in the soil network are gradually lost, which further threatens the aboveground biota, but also anthropogenic requirements, such as food provision and clean water.

In this thesis, we will focus on bacteria as they are arguably the single most influential group in the soil in terms of primary decomposition, nitrogen cycling and boosting plant productivity. We performed a microcosm experiment in which we manipulated a gradient of soil biotic complexity by consecutively filtering out taxonomic groups based on size from five contrasting grassland donor soils.

We found gradual changes in bacterial community composition as soil biotic complexity decreased, which was further represented in functioning. Relative abundance of nitrifying and denitrifying bacteria decreased significantly in less complex systems, while that of nitrogen fixers remained constant. Interestingly, this latter group had a much higher degree of redundancy, seeming to act as a safety net for the function within the ecosystem.

Further, plant productivity was impacted as herbs performed worse under reduced biotic complexity and root growth was increased, possibly indicating stress.

This study underlines the efficiency of a phylogenetically diverse soil system. Due to the disruption in the trophic and decomposition networks, the functioning of the overall soil network declines in its efficiency. This is alarming considering these belowground communities are the foundation of our aboveground world.

1. INTRODUCTION

The rapid development of human civilisation and our technology has been expressing itself in natural environments for centuries, building on the effects of anthropogenic geographic expansion of the last 50.000 years¹⁻⁵. Anthropogenic emissions, land-use change, over-fertilisation, pollution and overexploitation of natural resources are all leading causes of global change^{1,5,6}. While all of these different threats have their own specific effects on the natural world, one factor is affected by all: Biodiversity.

Species and genetic diversity are disappearing at an alarming rate, with some experts claiming we are headed towards the 6th great extinction event^{2-4,7-9}. These changes are often irreversible and will cause ecosystems to drastically change as their structure brittles. In some cases, a new balance is found where the dynamics are virtually the same as their natural conditions. Most times however, the disappearance of just one species in the network implies the extinction of many others that are directly or indirectly impacted by it, with the end result being that a minority of species dominate the landscape. Worst-case scenario, a biodiverse ecosystem will fall to dust into a barren wasteland.

Biodiversity is arguably the planet's most fascinating characteristic, but also its most precious resource^{10,11}. A highly biodiverse system performs more ecosystem functions and performs them at a higher efficiency compared to less biodiverse systems¹²⁻¹⁵. These functions maintain the stability of the ecosystem and the provision of exploitable resources, such as potable water, wood and produce¹⁴⁻¹⁹. Understanding and quantifying the dynamics behind ecosystem functions are valuable for improving human health and research efforts. The direct drivers of ecosystem functionality are biotic interactions which construct the species network^{15,16,20-22}. A plethora of studies has indicated the importance of even a single species or functional group for the overarching ecosystem they belong to. Apex predators, such as the grey wolf keeping elk populations low so riparian vegetation can recover in order to safeguard river systems^{23,24}. Seed-dispersers, such as the cassowary, are believed to play a large role in the rejuvenation of Australian tropical forests as they are crucial for the dispersal of over 70 different plant species^{25,26}. Animal pollinators, such as solitary bees, contribute across 200 countries to the production of 87 global crops, of which a third participate in global economic food production¹¹. But perhaps most impressive of all - performing multiple functions which are detrimental on a global scale -, right under our feet: soil microbes.

The majority of people, even amongst scientists, would not consider including the latter on their listing of examples. This is also very evident in ecological research, where soil microbiology is most often overlooked. Unfathomable to think, as soils are the most ubiquitous, varied and biodiverse ecosystems on the planet. Since the beginning of the century, appreciation for soil ecosystems has slowly been growing, with a general belief that soils contained 25% of the planet's biodiversity²⁷⁻²⁹. This estimate

has been largely misinterpreted however, as it was specifically made for animals, not all life forms²⁹. Anthony et al. (2023) re-estimated that 59 ± 15 % of all species, between 9.5 million and 160 billion, find their home in soils³⁰. This includes plants, macro- and even megafauna, but in this study, we will investigate the importance of the smallest and most diverse soil biota, namely soil microbes, microfauna and mesofauna.

The soil ecosystem

A myriad of functions is being performed by these belowground organisms in the soil system, including organic matter fragmentation and decomposition, nutrient cycling, plant growth promotion, water retention and carbon sequestration^{31–35}. All functions that many of us take for granted, but all play their part in human welfare, biosphere integrity and the fight against global change.

A general consensus is that many of these functions are overrepresented in soils, so called functional redundancy^{36–39}. The implication is therefore often made that the loss of a minority of soil taxa is of low consequence for ecosystem functionality as there are many other taxa that co-perform these functions⁴⁰. While this does hold merit, not all functions are being carried out at an even magnitude. The functions are often performed with different efficiency depending on the taxon and some specialist functions are not broadly represented at all by the taxa network^{36,41}. Studies have found that higher soil biodiversity increases the efficiency of the separate functions and increases multifunctionality by an even higher degree^{39,41–44}. This is indicative of the importance of interactions between the different taxonomic groups. **Soil biotic complexity** – soil community composition as expressed by the aggregation of multiple phylogenetically diverse taxa and their network of interactions– will be the main focus of this thesis.

During the course of their evolution, soil microbes, microfauna and mesofauna have created intricately interwoven networks between their representative taxa^{45–47}. Prominent representatives of soil microbes include bacteria, archaea, fungi and protists. The first three groups include primary decomposers – the conversion of organic compounds into inorganic compounds – and perform many other crucial functions in the soil ecosystem, with nutrient cycling being of relatively great importance and will therefore be one of the focal points in this thesis^{48–51}. Protists perform some of these functions as well, albeit to a lesser degree⁵². Their main influence in soil systems is arguably that of predatory protists, namely bacterial grazers, which have a top-down effect which will partly shape the structure of bacterial communities^{47,53–56}. Microfauna are represented at large by nematodes, whose trophic interactions on the prior groups have a varied range of top-down effects as they are often seen as primary and secondary predators in the soil food web^{57–62}. Lastly, the mesofauna that will be mostly taken into account in this thesis are collembola and mites, which cannot only acts as apex predators in the context of these three soil organism groups, but also benefit plant health as they can reduce pathogens and induce root growth for instance^{53,58,61,63–68}.

Naturally, the overview of these soil organisms and their interactions is incomplete and has been largely generalised for the purpose of clarity. We mentioned trophic interactions, but symbiotic and competitive relations are characterised abundantly as well ^{48,67,69–72}. The truth is that soil ecosystems are numerous in their biota and their ecology is specific down to species level, which means the interactions in the soil system are nearly impossible to track down, let alone to quantify^{31,73–78}.

In our experiment, we will attempt to create a biotic complexity gradient. Due to the relatively great importance of primary decomposers, supplemented with a range of highly specialised functions, this thesis will zoom in on bacterial communities with the addition of Archaea (but we will mostly vulgarise these together as bacterial communities). We will study on a broad scale how these communities are impacted by different complexity levels of soil communities and the implications for the soil ecosystem. In other words, what happens to bacterial communities in terms of composition and functioning if soil ecosystems are manipulated in such a way that major taxonomic groups are left out of the equation?

Bacteria in the soil

Bacteria are a crucial and large part of soil networks. Between 100 million and 1 billion individual bacteria can be found in just a teaspoon's worth of soil ⁷⁹. Ecosystem functioning in soils is arguably mostly influenced by bacteria, as their functioning is varied, with specific functions being linked to specific bacterial taxa ^{42,79–82}. We already mentioned that they act as primary decomposers. The other taxonomic groups of interest take part in the decomposition process as well, with fungi also playing their part in primary decomposition ^{48,83}. Micro- and mesofauna are important for fragmentation of organic material, making it more accessible for microbial decomposers. In essence, we have a conveyor belt on which organic material is passed on to different soil taxa which are specialised in the decomposition of organic matter of a certain size. Ultimately, primary decomposers convert organic compounds to inorganic form, making the nutrients accessible for organisms for biomass assimilation. Although, bacteria will incorporate most of these compounds directly into their biomass. Only through bacterial grazing (and the consumption of these grazers) will the inorganic nutrients be mineralised ^{47,58,61}. In systems where they are absent, bacteria will often monopolise these nutrients and largely immobilise them into their biomass.

Ecosystems therefore depend on decomposition and trophic interactions, especially for the accessibility of limiting nutrients, such as minerals, phosphorus and nitrogen. This last nutrient will be a main focus in this research to estimate dynamics of nutrient cycling in function of soil biotic complexity.

Nitrogen cycling as performed by Bacteria

Nitrogen (N) is the fourth most abundant element in biomass and the element that is extracted the most by plants from soils, an important resource to synthesise amino acids, nucleotides and chlorophyll ⁸⁴. While it is evident that N is an indispensable nutrient for all biota, it is often the most limiting resource

in natural conditions. Efficient Nitrogen cycling is thus necessary to support the productivity of the system.

An important result of global change is the impoverishment of soils, for instance due to large scale land-use change ⁸⁵. Nitrogen is further becoming an increasingly limiting factor due to the rising of atmospheric CO₂ stocks fertilizing the vegetation ^{86,87}. More biomass is produced wherefore more nitrogen is needed and extracted by plants from the soil. Nitrogen in these scenarios thus becomes even less abundant or accessible than ever before. This issue will be the primary threat focused on regarding nitrogen cycling in this thesis. However, the other extreme is perhaps equally prominent, especially in more anthropogenic landscapes and surrounding natural environments. Excessive nitrogen deposition, through fertilisers, have tipped the balance of many ecosystems ^{1,9}. Regardless, nitrogen as a limiting factor or as a pollutant, both extremes cause the loss of biodiversity. In suite, representation and efficiency of ecosystem functions are lost.

In nitrogen cycling, soil bacteria are amongst the most important biota, if not the single most determining group ^{51,88,89}. The most abundant inorganic form which is released by primary decomposers is ammonium, which plants are able to assimilate. This compound is further recycled by certain bacteria to a plant-available form via nitrification, which is defined by consequential ammonium and nitrite reduction, with the delivery of nitrate ⁹⁰. Nitrate is generally the preferred form of inorganic nitrogen for plant assimilation as ammonium is toxic in high quantities ⁹¹. Nitrification thus increases the efficiency of nitrogen cycling. Several Archaeal taxa also play a prominent role in nitrification ^{92,93}. A second process highly represented by bacteria is denitrification, which reduces nitrates in the soil to a gaseous form ^{94,95}. This implies that the contained nitrogen is generally inaccessible for most biota, meaning the process results in an outflux of nitrogen. Conversely, nitrogen fixers, free-living or plant symbionts, deliver an influx of nitrogen to the system as they are able to fix nitrogen gas, which is eventually made available to the soil system through decomposition ^{96,97}. This last process is especially crucial for plants in otherwise nutrient-poor systems .

Plant productivity as governed by soil biota

As mentioned previously, chlorophyll synthesis requires nitrogen. More nitrogen translates into more chlorophyll production, which will increase energy capture by plants, increasing their productivity and the fixation of carbon. This carbon supply will become accessible to the other biota of the system when plant matter is decomposed. However, plant roots actively release carbohydrates as well – exudates – these are small organic compounds, which can probe minerals loose from the soil and feed surrounding microbes ^{98,99}. The latter instigates the so-called microbial loop ¹⁰⁰. Bacteria will feed on the exudates in the rhizosphere and quickly grow in abundance, incorporating nutrients into their biomass. Through rapid depletion of these exudates, as well as bacterial grazing, these nutrients will become available in

the soil and accessible for plant assimilation. The microbial loop again showcases the multifaceted nature of the soil interaction network, as well as the importance of trophic interactions for soil fertility.

Plants thus supply the soil system with carbohydrates, boosting the performance of belowground biota. However, plant productivity in turn is governed tightly by soil biota, in part by decomposition and nutrient cycling, but even beyond these processes. Mycorrhizal fungi are plant symbionts which are in contact with the roots, expanding the reach of vegetation in the soil and thus allowing an increased supply of water and nutrients¹⁰¹. Collembola and mites can protect vegetation from soil-borne pathogens and induce root growth^{65–67}. Bacterial grazers such as nematodes and protists can prohibit nutrient immobilisation by bacteria for instance and improve mineralisation rates. Bacteria clearly play their part in this as well, with some of the crucial players being the nitrifiers and nitrogen fixers mentioned before.

The productivity of vegetation is often quantified by biomass production. Aboveground biomass in particular reflects the competitive ability and overall performance of individuals and/or plant species. Root biomass reflects to a certain degree also the performance of vegetation, but can also be an indicator of the level of stress the vegetation experiences¹⁰². Many plants create vast root systems in order to meet their nutritional needs, while vegetation in nutrient rich systems often shows limited root growth as nutrients are not limiting and the plants can invest more into their shoot.

Since there are many different ecosystems, all with their own characteristics in plant communities, we have selected one that is of especially great importance as it is ubiquitous both in natural and anthropogenic landscapes: grasslands.

Grasslands as a model ecosystem

Grasslands cover close to one third of the earth's terrestrial surface and a third of Europe is also dominated by grasslands and rangelands^{103–105}. Grasslands are natural or semi-natural landscapes with vegetation dominated by grasses, sedges, herbs and rushes. European grasslands are a refuge for many rare and threatened species, including birds, butterflies and plants. Understanding how soil biotic complexity influences these ecosystems is thus an invaluable asset in conservation efforts, yet also for agricultural services, in which many grasslands play a vital role through livestock grazing^{106,107}. Grasslands are also generally valued by the ecosystem services they provide, including carbon storage, ground water replenishment and protection against erosion^{107,108}. Being often linked with agricultural systems, many grasslands are under threat of nitrogen deposition¹⁰⁵.

Grasslands can differ in their characteristics, such as soil texture, pH, C/N ratio, (plant) species richness and overall productivity^{104,105,109}. These intrinsic characteristics can already shape soil communities through mechanisms we have briefly touched on before.

In this research, we want to acknowledge the fact that bacterial communities are in part shaped by these characteristics, which leads us to believe that different grasslands might show different reactions to the

induced soil biotic complexity gradient. We thus research the importance of soil biotic complexity in different grassland types. We were especially interested in the common responses found across multiple grasslands, but also examined grassland responses to broaden our understanding of interactions between soil biota and their environment.

Knowledge gaps in soil ecology and perspective of this research

The role of soil organisms is evidently a crucial one and research in the field has increased greatly in the last decade, as 2020 saw a sixfold in the number of publications on the soil microbiome compared to 2010³¹. Yet today, still relatively little is known about soils and inhabitant biota. Amongst studies that in fact study soil microbiology, a high priority is given to Bacteria and Fungi. Mishra et al. (2023) found that 55% of published soil microbiome studies between 2005-2021 (n= 7434) studied Bacteria, 30% studied Fungi, with the remaining 15% being further divided into Archaea, mesofauna, algae and protozoa³¹. Meanwhile, the meta-analysis of Guerra et al. (2020) found that only 0.3% of sampling sites of soil research (n=3.318) have an overlap on both soil biodiversity and functional datasets, with a non-systematic coverage of just a few taxa and functions across sites¹¹⁰.

Especially the interaction network within the soil is still under great question. While a lot of research has been done on interactions between a few specific species or the effect of one species on a larger taxonomic group, for instance bacteria, there is a knowledge caveat regarding the larger picture. While the findings of these studies are of great value in better understanding the network, it is arguable that these effects will be represented in natural conditions the way they are in these experiments. The experimental systems are extremely simplified and have only a fraction of the interactions that natural systems do. I argue that some of the researched biota are therefore experiencing ecological release, in the sense that they are able to perform better due to the absence of any notable competitors or predators. Conversely, I believe other taxa will perform much worse due to the lack of their symbionts or taxa they usually depend upon (in)directly for the creation of their specific niche. I therefore argue that any results of these experiments will be much more or much less pronounced than they would in natural conditions where a myriad of different biota affect each other.

The goal of this experiment is to shed light on the importance of soil community complexity, how different levels of complexity affect bacterial community composition and the implications on ecosystem functioning.

Our experimental design manipulated belowground soil biotic complexity through inoculation of sterilised grassland microcosms in which we observed the interactions between bacterial, general soil biota and plant communities. Four different complexity levels have been created: Max, High, Low and Min. Each name is an indication of the relative complexity compared to the other levels. This was achieved by sieving donor soil sequentially through different mesh sizes, which has been empirically proven to be able to successfully filter out specific taxonomic groups in their entirety^{111,112}.

Since the varying characteristics of grasslands co-determine composition of soil communities, five different European grasslands have been selected to act as donors for this experiment. This allows us to pinpoint what patterns are generally observed for grasslands and which patterns occur for specific grasslands (and thus different conditions).

After the experiment had been conducted, plant biomass was gathered to study plant productivity and soil samples were taken to extract bacterial DNA in order to analyse the bacterial community composition. Through bio-informatics, this latter data was also used to procure information on the potential bacterial functionality regarding nitrogen cycling.

Research questions and Hypotheses

1. Does soil biotic complexity affect the bacterial communities of grasslands and how?

I hypothesise that bacterial communities will become less diverse as soil biotic complexity decreases. I further expect that there is a gradual change in which relatively more complex systems will contain more similar bacterial communities compared to less complex systems.

I propose top-down regulation as a main process in shaping bacterial communities, which will differ in soil communities of different biotic complexity. An increased predative pressure, but also an increased competitive pressure will boost bacterial diversity. Additionally, a more complex soil biotic network will create more differentiated niche opportunities, further boosting bacterial diversity.

I expect that the absence or presence of these effects will create a general pattern across the grasslands, differing in relative magnitude due to abiotic and biotic differences.

2. Does soil biotic complexity affect bacterial functioning in grasslands, specifically nitrogen cycling?

I hypothesise that bacterial nitrogen cycling will decrease in its efficiency as soil communities decrease in their complexity.

I expect potential nitrification, denitrification and nitrogen fixation to decrease as the complexity of the soil biotic system decreases. Especially nitrification and nitrogen fixation are key functions in the soil system, implying that nitrifiers and nitrogen fixers form important nodes in the biotic soil system. As these biotic networks degrade with a lower soil biotic complexity, I hypothesise that the performance of these bacteria will decline prominently. For nitrification, I expect that the disruption of the decomposition network will be the leading factor of its decline.

I expect a less pronounced change in potential denitrification as denitrifying bacteria form more of a decoupled niche, with few interactions between them and other biota.

I will research the level of redundancy of these functions in the system and try to link this to their level of stability across the complexity gradient.

3. *Does soil biotic complexity affect plant productivity? How is this reflected in aboveground (AGB) and root biomass?*

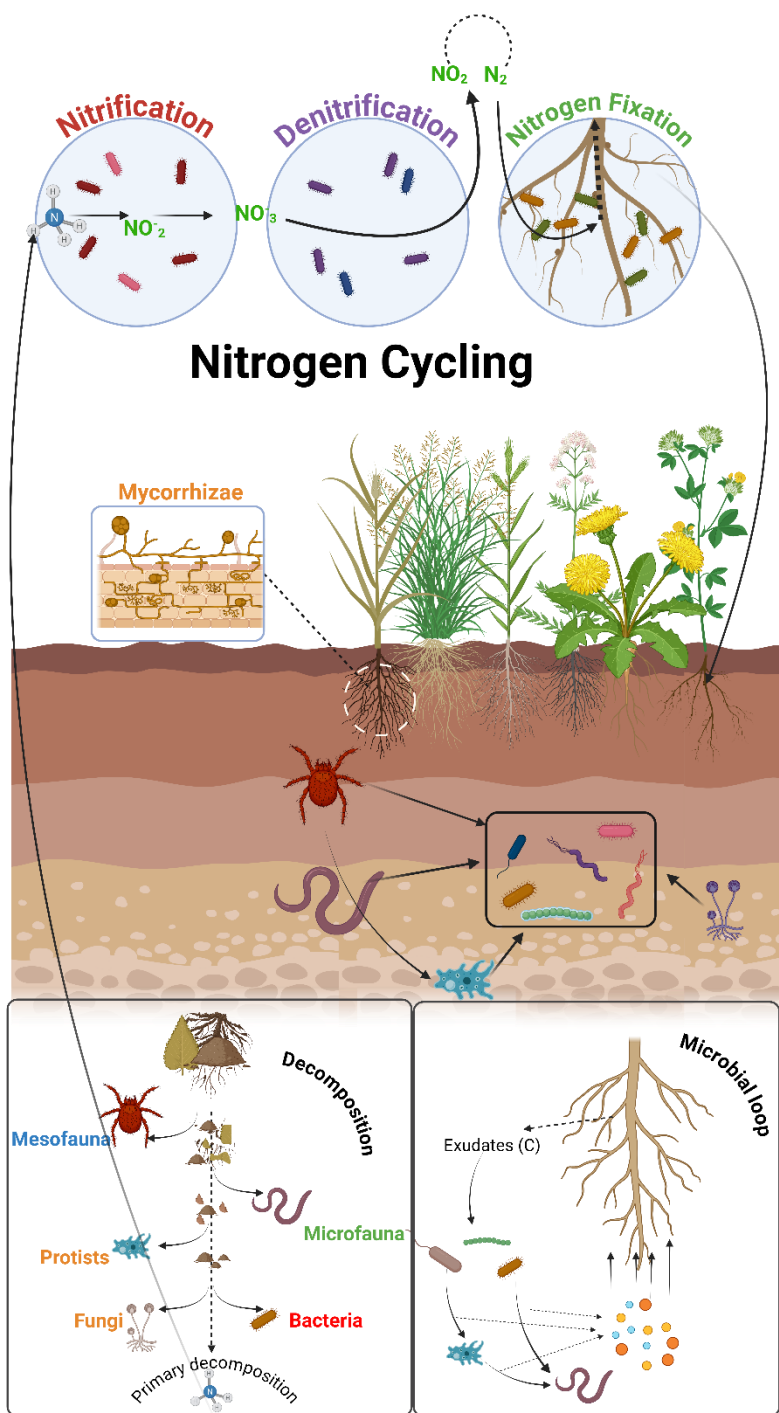
I expect that AGB will decrease with decreased soil biotic complexity in response to a decreased efficiency of nutrient cycling, especially pronounced when mycorrhizae are excluded from the system.

I expect a disproportionate decrease in herbs compared to grasses as they depend more on these symbionts.

I hypothesise that root biomass will decrease when mesofauna are excluded from the soil system as they promote root growth. However, I expect a net increase compared to the most complex systems when mycorrhizae are excluded. The latter being a stress response due to a decreased efficiency of nutrient assimilation.

Please refer to Figure A for an illustrated overview of the hypothesised dynamics between highest and most reduced soil biotic complexity.

High Soil Biotic Complexity



Low Soil Biotic Complexity

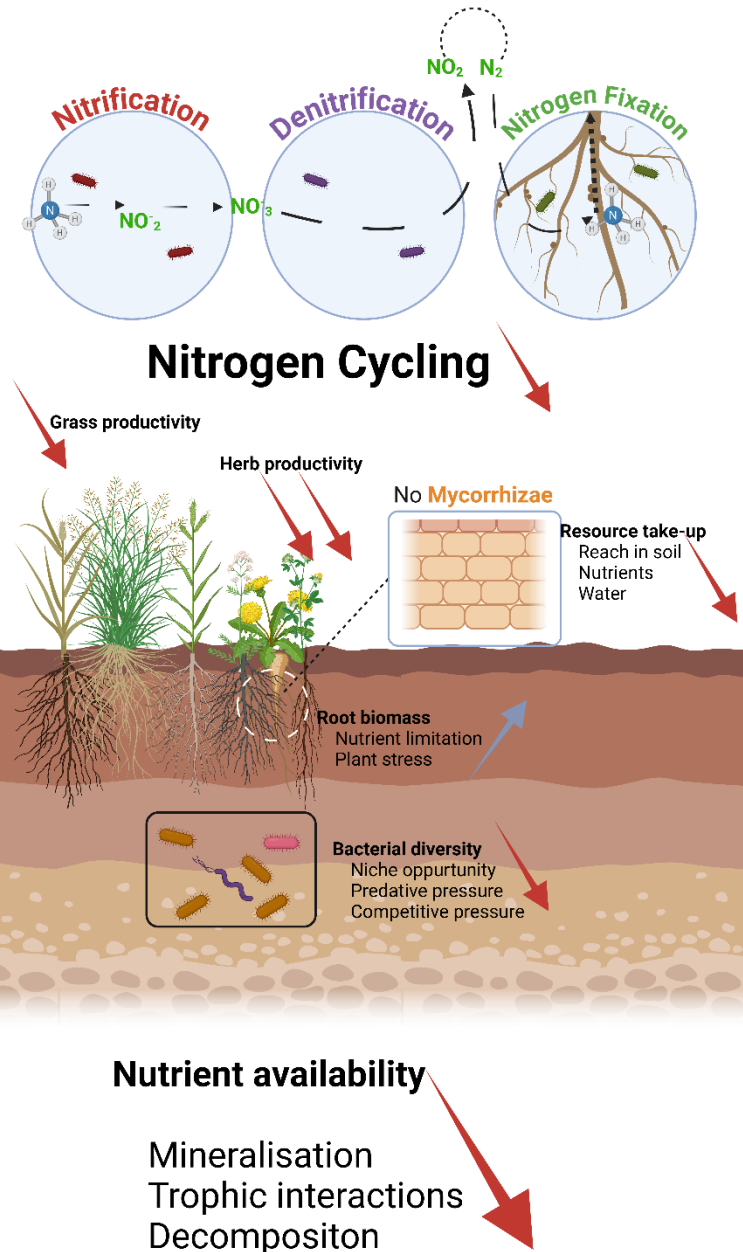


Figure A. Soil biotic community hypotheses visualisation. This scheme shows the two most contrasting scenarios in the soil biotic complexity gradient. **Left** is the natural scenario depicted with all functions and interaction networks in their optimal state. The main figure shows a simplified trophic network and top-down dynamics in regards to the bacterial community, as well as competitive pressures. **Right** depicts the most reduced scenario that will be regarded in this thesis with the expected responses of bacterial communities, Bacterial nitrogen cycling and plant productivity. The **Decomposition** and **Microbial loop** panel highlights networks and dynamics which are absent in the reduced scenario. For further explanation of the different dynamics and hypotheses, refer to the introduction and research questions.

Soil organism names and the two scenario headings were colour coded to comply with the complexity levels which will be further discussed in methodology. Colouring of nitrogen cycle functions is purely aesthetic. Scheme was created in BioRender.com

2. METHODOLOGY

2.1 Experimental setup

Soil collection

Soils for the experiment were collected from five different grassland across Europe, differing in characteristics (see Table A). Soil collection was performed in late summer/autumn 2022 (for GER, IT, HD and HW) and early spring 2023 (BZ). Approximately 80 kg of soil was collected per site which consisted of the upper 20cm topsoil in. Soil collection was performed on an area within the grassland site within the bounds of 5m², after which it was mixed in order to get a uniform sample in terms of substrate and biotic community. The soil was translocated to the University of Antwerp and stored until the beginning of the experiment. To keep the soil communities sustained until that period, the soils were moistened to prevent desiccation and plants were allowed to maintain growing.

From the selected soils, a subsample was taken to extract inoculum and to assess soil properties. The remaining bulk of soil was designated to act as an almost completely sterilised growing environment for the manipulated microbial and plant communities. For biotic suppression, the soil was placed in aluminium trays (depth: ~10 cm) and left in a drying oven at a temperature of 105°C over a time period of three hours, which was repeated three times. Resting phases were at room Temperature and lasted 48 hours. The first cycle had matching aluminium lids shutting off the trays, which induced so-called “steam sterilisation” and were then put to rest for any remnant soil communities to develop, making suppression afterwards more effective . Then the second cycle commenced, this time with lids taken off in order to dry out the soils. After another resting period, the third cycle commenced with the trays once again sealed shut and stored away until the beginning of the experiment. We are aware that this technique could allow a minority of spore-forming bacteria to survive, but the vast majority of biodiversity will be eliminated.

Table A. Overview grasslands. The grassland codes will be used to refer to specific grasslands within the thesis. Characteristics were provided by local managers and are therefore expressed in more broad terms.

<i>Grassland code</i>	<i>Land of origin</i>	<i>Latitude</i>	<i>Longitude</i>	<i>Characteristics</i>
IT	Italy	42.95587	13.01711	Sub-Mediterranean calcareous montane grassland, species rich and nutrient rich, with loamy soil and neutral pH
GER	Germany	54.06019	12.23444	Temperate mesotrophic grassland, more acidic with sandy colluvial soil
HW	Hungary	47.40046	19.15883	Temperate wet meadow, mesotrophic, species rich, with loamy soil and neutral pH
HD	Hungary	47.39967	19.1533	Temperate, oligotrophic, sandy grasslands with xerophilous vegetation, more alkaline
BZ	Belgium	51.19323	4.943781	Temperate mesotrophic grassland with clay-loam soil and neutral pH

Manipulating microbial complexity

Four different complexity levels were created by sieving the previously subsampled soil through different mesh sizes. 250 g of soil was dissolved in 0.5 L of distilled water, which was consecutively sieved through different mesh sizes depending on the desired complexity level. See Table B for a detailed overview of the contained taxonomic groups per Complexity level, as well as the different sieve/mesh sizes used to manipulate the community composition. Since there are 5 grasslands and 4 complexity levels, this added up to 20 unique inoculum types. Each type was independently replicated by 10, for a total of 200 inocula. Inocula were freshly prepared right before inoculation.

Table B. Overview complexity levels. Complexity level refers to the different treatments in the experiment, characterised by the mesh size used to create the designated inoculum. Largest taxonomic groups is the broad group present in the complexity level which would be filtered out in the sequential step. Taxa of interest summarises briefly the few specific taxa we will regard in this thesis to form the discussion on regarding the effect of treatment, even though many more taxa will be present in the microcosms. Complexity levels are colour coded. These colours will be used throughout the thesis. Complexity level Max will often be the reference point as it most closely resembles natural conditions, while the remaining three levels together will be referred to as reduced complexity levels. Keep in mind that in essence, all treatments are reduced in comparison to natural conditions.

	<i>Complexity level</i>	<i>Mesh size</i>	<i>Largest Taxonomic Group</i>	<i>Taxa of interest</i>
<i>Most natural</i>	Max	2 mm	Mesofauna	Collembola + Acari
<i>Reduced</i>	High	150 µm	Microfauna	Nematodes
<i>complexity</i>	Low	20 µm	Protists + Fungi	Protists + Fungi
<i>levels</i>	Min	2 µm	Prokaryotes	Bacteria (+Archaea)

Plant community selection

Per grassland site, assessments in the field were made of the vegetation with a focus on which plant species were relatively dominant. Estimations were made in the grassland sites by local managers. A total of 10 dominant plant species were chosen, with an ideal distribution kept in mind: 5 grasses (mostly poaceae) and 5 herbs. Seeds matching the native species were purchased from commercial suppliers, unless they were unavailable. This was done for the majority of plant species, but in this case they were unavailable, seeds were collected from the field in their native grassland site. Germination rates of seeds were tested in petri dishes with a moistened paper pad and most were generally successful for most species. Ultimately, the goal was set to have at least six plant species per plant community in which both grasses and herb species were represented per grassland site.

Seeds were surface sterilised by washing them with diluted bleach (4%) for 1 minute, after they were rinsed with 70% ethanol and lastly distilled water. Doses of seeds were prepared per microcosm in such a way that each microcosm of a certain grassland site got approximately an identical mixture of seeds.

A similar volume of seeds was measured using small spoons of a known volume, where the relative proportion per species depended on the size of the seeds and the previously assessed germination rate.

Microcosms

In spring of 2023, the microcosm experiment commenced. Microcosms were placed together in one large greenhouse on tables which were covered in plastic foil to create a sterile barrier between the microcosms and the greenhouse environment.

Each unique inoculum created before was designated to be used for one microcosm of the corresponding grassland x complexity level combination. 200 inocula were thus prepared for 200 mesocosms, which were set up together in a single large greenhouse. Pots were used with the following dimensions: diameter=25 cm and height=20cm. These pots were cleaned with bleach before they were filled with a 10 cm layer of sterilised extended clay pebbles, which were put in place to allow adequate drainage of excess water. Additionally, since the amount of donor soil was limited, this choice allowed the vegetation to expand their roots further as larger pot sizes could be used. The remaining volume of the pot was filled with a mixture consisting of donor soil (1.2 kg), sand (400 g) and clay pebbles (500g). The latter two substrate types were sterilised through thorough washing followed by three hours in the drying oven at 105-120 °C. The sand substrate was chosen to be coarse river sand, which typically contains a minimal amount of nutrients. Once set up in March 2023, the inoculum was added and allowed to establish for 5 weeks with minimal contamination by sealing the microcosm off with disinfected plastic foil. Due to the work load of this process, microcosms were set up per grassland site on the same day, with two days left in between two grassland sites. A total time span of ten days had therefore passed from the first grassland site to the last grassland site.

The preparation of the Min inocula proved to be a time consuming task due to the small mesh size that was used. We estimated that an average of 100 g of soil was supplemented to the microcosms in this initial attempt. The choice was made to supplement these Min microcosms with additional inoculum, again freshly prepared, after a period of 3 weeks of biomass recovery. In each treatment, the amount of soil which failed to pass through the filter was quantified and compensated with sterilised soil of the corresponding grassland site. This ensured that all microcosms were supplemented with approximately 250g of soil.

After a 5-week establishment period, any emerging sprouts were removed as they were the effect of inoculum addition (possible in Max due to the 2 mm mesh size) or in rare cases, resistance to high temperatures during biotic suppression. After, the 10 selected plant species per grassland site were sown into the microcosms. The seeds were distributed evenly over the soil surface and then covered by a thin layer of sterilised donor soil. After this process, the locations of the different microcosms in the greenhouse were randomised. Pots were placed in groups of five with all microcosms belonging to the same complexity level, but different grassland sites (one of each). Pots within a group were placed with

approximately 20-30 cm between them, while groups had approximately 50 cm between them. This way, grassland sites were randomly and evenly distributed across the greenhouse, preventing a local difference in environment to affect grassland site unevenly, while keeping cross-contamination risk at a minimum by keeping the different treatments more or less isolated from each other.

The plants were left to develop in the greenhouse for 18 weeks with natural light and were watered 2-3 times per week with filtered water (0.03 µm filter) to eliminate biotic and nutrient contamination. Watering frequency and volume was adjusted to the microcosm needs but were always kept the same per grassland site. The greenhouse had an automatic shading system in which lightly coloured blinds blocked extensive amounts of sunlight. Ventilation was also regulated by an automatic system in which ceiling windows were opened when a Temperature threshold of 30°C was breached. Nevertheless, an exceptionally warm and sunny summer, with temperatures reaching over 35°C imposed heat stress on the plants. This is presumed to have affected plant growth and final biomass productivity was lower than was expected under optimal conditions. Especially the performance of herbs was suppressed. Nevertheless, these conditions affected different treatments in a similar manner allowing for the effective comparison of plant growth between the treatments.

At the end of the experiment, plant biomass was harvested with the division made into aboveground biomass and root biomass. Aboveground biomass was cut at the surface and divided into grasses, herbs and litter if present. After, root biomass was harvested with as much of the soil washed away. An additional washing process was instigated to clear as much of the soil. Both the collection of fine roots and the complete removal of soil from the roots were difficult tasks and could both have lead to small biases. All forms of biomass were dried for 48 hours at 70°C and weighed (precision 0.001 g). All microcosms had established plant communities which could be analysed (n=200). Soil samples were also taken per microcosm at a depth of 0-10 cm. A smaller subsample was stored at -20 °C (for DNA analysis).

2.2 DNA extraction and sequencing

DNA extraction

DNA extraction was performed on 250 mg of soil for each sample, making use of the DNeasy PowerSoil Kit ® and following the prescribed protocol of the manufacturer (Qiagen, Venlo, the Netherlands). One modification of the protocol was made, a vacuum manifold was used in order to remove the washing buffers from the columns, rather than additional steps of centrifuging. DNA extracts were stored again at -20 °C for future analysis, which was repeated after all future steps.

Qubit

To assess if a sufficient amount of DNA was extracted in the previous step, a sample size of at least 3 samples per complexity level of each grassland site was selected for analysis with Qubit®. The Qubit® 3.0 Fluorometer (ThermoFisher Scientific, Massachusetts, USA) was used to assess DNA concentration fluorometrically.

The DNA extracts of grassland site HD had an optimal concentration. Concentrations of the following grassland sites were deemed too high to ensure the quality of PCR: BZ, GER, HW and IT. Therefore, $\frac{1}{3}$ dilutions were made of these DNA extracts. In future reference, the term DNA extracts will be used out of convention for both diluted and undiluted DNA extracts.

PCR

For identification of the bacterial communities, the bacterial 16S V4 region was targeted and amplified using the 515F-806R primer pair (Caporaso et al., 2011), supplemented with Illumina Nextera adapters (ThermoFisher Scientific, Massachusetts, USA). Two rounds of Polymerase chain reaction (PCR) were performed with a negative control per batch to keep track of possible contamination. Gel electrophoresis was performed as a quality check after each round.

Negative controls

A negative control was added to each batch of samples undergoing PCR. For them, the volume of the sample was interchanged by additional nuclease-free water in order to keep concentrations equal.

PCR I

For PCR I, Reaction mixtures of 25 µL were prepared in a sterile UV cabinet with products from the Phusion® High-Fidelity Kit (New England Biolabs, Ipswich, United Kingdom). Each mixture contained: 1 µL DNA extract, 17.3 µL nuclease-free water, 1x Reaction Buffer, 200 µM dNTPs, 0.2 µM forward primer, 0.2 µM reverse primer and 1 U DNA polymerase. The C1000 Touch™ Thermal cycler was used for PCR.

PCR I conditions were as followed: initial denaturation at 98 °C for 60 s, followed by 25 x (denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s) and an additional extension of 72 °C for 10 min. After the program was done, samples were kept at 10 °C in an infinite hold until taken out of the machine.

PCR II

Successful PCR I products were diluted 50-fold and PCR II was performed. Reaction mixtures (total volume of 25 µL) consisted of 2 µL diluted PCR I product, 12.3 µL of nuclease-free water, 1 x Reaction buffer, 200 µM dNTPs, 1 U DNA polymerase and 5µL of a unique primer with Illumina adapters and library indexes. The conditions were: 98 °C for 60 s, 12 x (98 °C for 30 s, 63 °C for 30 s, 72 °C for 30

s) and 72 °C for 5 min. After the program was done, samples were kept at 10 °C at infinite hold until taken out of the machine.

Gel electrophoresis

Quality control via gel electrophoresis (1.5% agarose in 1 x TAE buffer) was performed. PCR products were stained with Midori green, a fluorescent DNA binding dye, and ran on the gel for 40 minutes at 90 V. To assess if PCR was successful, we compared samples with a reference ladder and confirmed if the 480bp band was present, which is an indicator for bacterial DNA.

Sample pooling Library preparation

Since band intensity was uniform across all PCR II products, 10 µL of the products of all samples, negative controls and technical repeats were pooled in a single 5 mL Eppendorf ®. The pool was thoroughly vortexed to create a homogenised mixture, after which 10 µL was extracted to perform gel electrophoresis in the same manner as described above. The band of interest was cut out of the gel, omitting primer dimers and other types of contamination.

The libraries were purified using the Machery-Nagel PCR cleanup and gel extraction kit (Machery-Nagel, Düren). DNA concentration of this pooled library was assessed via the Qubit ® protocol mentioned above and was equal to 19.4 ng µL⁻¹. The final concentration of the library was determined by performing qPCR using the KAPA library quantification kit (Roche, Bazel) before it was diluted to 4nM and sent to the Centre of Medical Genetics of the University of Antwerp for Illumina MiSeq sequencing. A total of 212 samples (200 unique, 6 technical repeats, 6 negative controls) were sequenced for 300 bp, both for forwards and reverse reads. This supplied us with FASTQ files, which contained all DNA sequences per sample.

2.3 Bioinformatics

Rstudio (version 2023.12.1) was used, which draws on R (version 4.3.2) (2023-10-31 ucrt) ([R Core Team, 2023](#)).

The FASTQ files were cleaned, merged and filtered using the R package dada2 (v.1.16.0)(Callahan et al 2016) and the accompanying dada2 pipeline was followed. Adjustments to this pipeline were the following:

- truncation was set to (F = 270, R = 200) as respective quality scores remained overall optimal at these thresholds.
- Quality filtering (maxN = 0, truncQ = 2, maxEE = 2)
- Merging (min overlap = 50 bp) to allow a high quality of merging forward and reverse reads

The resulting output was an Amplicon sequence variant (ASV) table with 10.921 unique ASVs.

ASVs were assigned taxonomic information by use of the SILVA database (version 138.1) and the DECIPHER Bioconductor package (version 2.30.0) ¹¹³. The output was a taxonomy table which holds taxonomic information for all ASVs from kingdom to species level (with NA values for unidentified taxonomic levels). By matching the ASV and taxonomy table, all unidentified ASVs were filtered out of the ASV table (10.921 total reads; so unchanged). Next, mitochondrial, chloroplast and eukaryotic reads were removed from the dataset (10.499 total ASVs). Archaeal reads were kept to give a broader overview of functioning (since they are known to contribute to N cycling), while having a low impact on community composition due to their overall low proportion in the dataset (Archaea made up <0.33 % of reads in the final ASV table). Out of convention, we will always refer to these prokaryotic communities as bacterial communities.

To be able to compare samples with each other, rarefaction curves were checked together with the total reads per sample. Based on this information, rarefaction was performed at 6587 reads (10.082 total ASVs). In total, 13 samples failed to meet this threshold, due to failure at one of the previous stages, and were cut from the analysis all together. An overview of the number of remaining samples can be found in Appendix 1. Enough samples were left for each complexity level per grassland to perform further analysis. All technical repeats closely resembled the original sample and were thus filtered out (total ASVs 10.002). Lastly, negative controls were taken out. The final ASV table consisted of 9.915 ASVs.

The negative controls for BZ, GER, IT and HW were between 0-311 reads, while the negative control of HD contained 1536 reads. Closer inspection of the two most represented ASVs in this last control ($n > 700$ and $n > 100$) showed that these were both completely absent in all samples of the corresponding grassland site. Considering this and the fact that all total reads of negative controls were substantially below the rarefaction threshold indicates that possible sample contamination was of no or low importance.

2.4 Statistical analyses

All statistical analyses were performed using Rstudio (version 2023.12.1) , which draws on R (version 4.3.2) (2023-10-31 ucrt) ([R Core Team, 2023](#)).

The following packages were used for analysis: vegan (version 2.6 – 4) , microeco (version 1.6.0), nlme (version 3.1 – 162) , lme4 (version 1.1-35.1), multcomp (version 1.4 -25), pairwiseAdonis (version 0.4.1), R6 (version 2.5.1), stats (4.3.0), emmeans (version 1.10.2)

The primary goal of analyses was to test general patterns across all grassland sites. In general, grassland site was thus considered as a random effect. However, we were also interested in how a range of different grassland characteristics can express themselves in bacterial and plant communities. Therefore, additional tests were run for some analyses to discern patterns for the specific grasslands (“case studies”) to research the degree of divergence between patterns across grasslands.

Bacterial community composition

Alpha diversity

Alpha diversity of bacterial communities was assessed by ASV richness and diversity (Shannon-Wiener index), respectively with the `specnumber` and `diversity` functions of the `vegan` package.

For general analysis, linear mixed effect models were constructed with the `lme` function of the `nlme` package. Alpha diversity parameters were tested in function of complexity level with grassland site as random effect.

For case study analysis, linear models were used per grassland with the `lm` function of the `stats` package, testing the alpha diversity parameters in function of complexity level.

Beta diversity

Beta-diversity was first assessed by multivariate analysis making use of functions of the `vegan` package (unless stated otherwise).

General analysis was performed as follows:

To visualise the similarity of bacterial communities, a multivariate ordination was made. The ASV table was standardised using the `decostand` function with “Hellinger” method selected. Next, a distance matrix was made using the `vegdist` function with `method = “bray”`. This distance matrix was used to perform a distance-based redundancy analysis (dbRDA) using the `dbRDA` function which was constrained on grassland level in order to more closely compare the complexity levels with each other. The plot was visualised using the `ggplot2` package.

To test for significant differences in community composition across the complexity levels, a PERMANOVA test was performed using the `adonis2` function on the ASV table in function of complexity level with grassland site as strata (`method=“bray”`, `number of permutations=9999`).

Case study analysis was performed per grassland site as followed:

A multivariate ordination was constructed, this time using non-metric multidimensional scaling (NMDS) via the `metaMDS` function. Plots were visualised using the `ggplot2` package.

After, the pairwise.adonis (package pairwise.adonis) function tested the pair-wise comparisons for complexity levels (sim.method=bray, number of permutations=9999).

Dominant taxa of bacterial communities

To discern shifts in major taxonomic groups, the 10 most abundant phyla and the 10 most abundant genera in the dataset were selected for analysis.

First, to assess the taxonomic assignment rate on phylum and genus level, the number of reads were summed for all ASVs which had been assigned a respectively a phylum and a genus. These sums were then divided by total number of reads within the ASV table. Further, to assess the 10 most dominant phyla and genera, reads were aggregated on the corresponding taxonomic level, showing per phylum and genus the number of reads within the dataset. Proportional values were calculated by dividing the aggregated reads by total number of reads. Based on this table, the 10 most abundant representatives were selected.

Linear mixed effect models were run per phylum and genus to discern general patterns in order to give a broad indication of whether the relative abundance of dominant bacterial taxa was impacted by differences in complexity level. The lme function (nlme package) was used of with reads in function of complexity level and grassland site as random effect. Adjustment of p values was performed using the Benjamini-Hochberg method to correct for multiple testing separately for the two taxonomic levels (10 tests with 4 parameters per test). See appendices 7 and 8 to see the relative abundances of these phyla and genera per sample.

For genera, a brief literature study was carried out to see how the general functioning of the bacterial communities could be impacted due to these shifts.

Ecosystem Functioning

Nitrogen Cycle

The R package “microeco” was used for the analysis of the nitrogen cycle functions. Through this package, the functional prokaryotic database FAPROTAX (version 1.2.6) was accessed ¹¹⁴. Based on the taxonomic information obtained through the SILVA database, FAPROTAX assigned functions based on literature to the different ASVs. FAPROTAX compiles reads per function per sample. The microeco package converts these reads into relative abundances (%).

The “Tutorial for R microeco package” was used as a basis, notably chapters 7.2 and 14.9 ([Tutorial for R microeco package \(v1.7.0\) \(chiliubio.github.io\)](https://chiliubio.github.io/microeco/tutorial/)).

We reported the percentage of ASVs that were assigned at least one function as an indication of the knowledge gap regarding the entire dataset. We analysed the following functions: nitrogen fixation,

nitrification and denitrification. Nitrification is a generalised function provided by FAPROTAX which compiles ammonium and nitrite nitrification.

The number of total ASVs that performed the three different functions was reported, as well as the mean ASV number per complexity level per grassland site.

Through generalised mixed effect models (glmer from the lme4 package; family=poisson), it was tested if the representation, in terms of unique ASVs, of the three different functions was impacted by the level of biotic complexity. All models were tested for overdispersion by dividing residual deviance by the degrees of freedom. All results were <1 .

Through mixed effect models (lme function), we assessed if there were general differences in the relative abundance of bacteria performing these functions (grassland site as a random effect).

For the case study analysis, generalised linear models were used for the ASV representation and linear models for the relative abundance. The pchisq function was used to test overdispersion of all glms. None of the models showed overdispersion.

Improved models

Upon final revision of the thesis manuscript, the lme tests performed in section *Dominant taxa of bacterial communities* and *Nitrogen Cycle* (relative abundance) were deemed to not be the most suitable models. Due to time constraints, they were not adjusted at this moment in time, but will be in the future prospects of this study.

We would use the glmmTMB function of the glmmTMB package (version 1.1.9) to fit a mixed effect model analogous to the lme function. For the dominant taxa, the rarefied ASV reads would be divided by the rarefaction threshold (6587) in order to get a relative abundance value, after which the logp function is used to normalise residuals and account for 0 values. The inverse logit of this value is taken to counteract the family “logit” in the glmmTMB function.

A preliminary model has been run for the dominant genus *Thiobacillus*, which had a model output comparable to the lme model. Therefore, we defend the results of these models, although acknowledging that they are not the most appropriate choice and that there might be minor differences in coefficients and p values. Most p values for these models were highly significant, indicating that the underestimation of p values probably caused minimal bias.

Plant Productivity

The aboveground and root biomass was analysed through mixed effect models in function of complexity level and grassland site as a random variable. The aboveground biomass variables analysed are: general aboveground biomass (AGB), grass biomass, herb biomass, litter biomass and herb:grass biomass ratio. Belowground biomass variables analysed are: root biomass and root:shoot biomass ratio.

Linear models were used to assess these differences in specific grasslands.

2.5 Figures

All figures were made using the packages ggplot2 (version 3.5.0) with multi-panel figures put together using ggpubr (version 0.6.0). Inkscape was used for some stylistic adaptations (Inkscape Project. (2020). Inkscape. Retrieved from <https://inkscape.org>).

3. RESULTS

Statistical significance was not reported for all models in the main results. Remaining statistical differences are describes in Appendix in section “Supplement model outputs”.

3.1 Bacterial communities

Alpha Diversity

Alpha diversity of bacterial communities in the mixed model showed significant differences in both ASV diversity and ASV richness (See Figure 1).

ASV richness was found to be significantly increased in complexity level Low compared to Max ($t_{179}=2.478168$; $p=0.0141$). However, the post-hoc Tukey test finds only a trend for the pair-wise comparison between Max and Low ($z=2.478$; $p=0.0635$), while the pair-wise comparisons for Min do find that its ASV richness is decreased compared to Low ($z=-2.786$; $p=0.0274$) and High ($z=-4.163$; $p<0.001$).

Shannon-Wiener Index diversity was significantly lower in complexity level Min compared to Max ($t_{179}=-4.36485$; $p<0.0001$). Post-hoc Tukey test affirms that ASV diversity in Min is lower compared to all three other complexity levels all characterised by a common p-value threshold (Max: $z=-4.365$ | High: $z=-4.718$ | Low: $z=-6.054$; $p<1e-04$). All other differences were not significant.

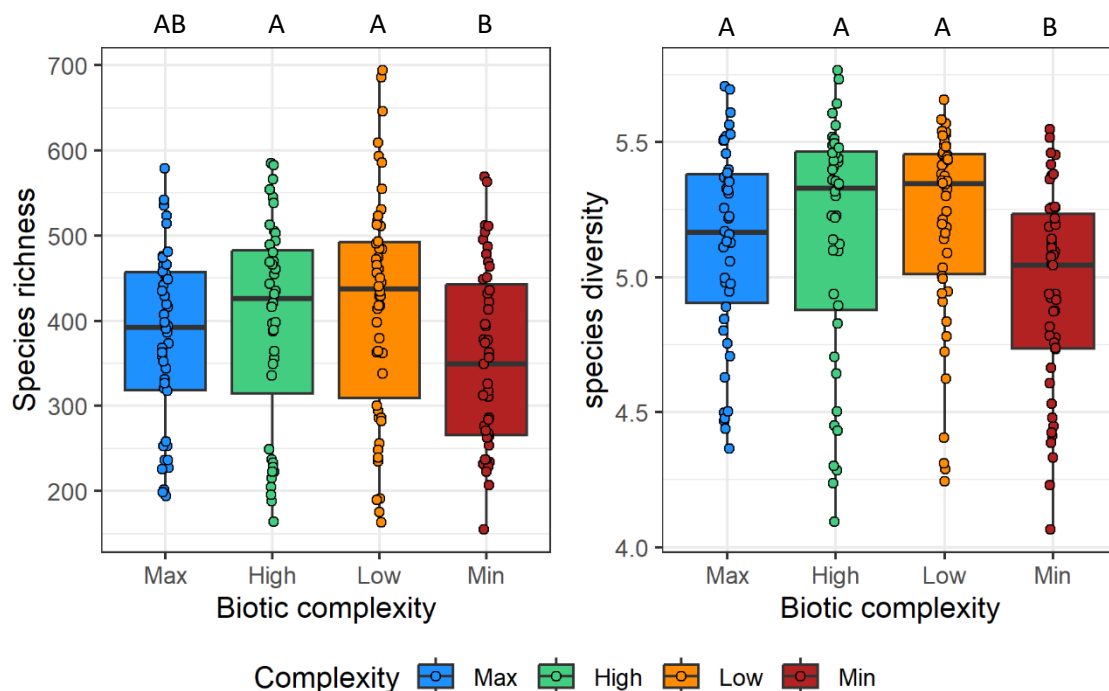


Figure 1. Alpha Diversity across the Complexity levels. This figure shows the variation in species richness (Left) and Species diversity based on Shannon-Wiener Index (Right) for each complexity level regardless of donor grassland. Significance letters are based off a post-hoc Tukey test mentioned in the text, with equal letters signifying no significant difference, while different letters signify a significant difference between two levels.

Bacterial communities of different grasslands differed substantially in alpha diversity across the biotic complexity gradient (see Figure 2 for diversity index). Overall, two grasslands (BZ-GER) showed a decrease in alpha diversity (richness and diversity index) compared to Max. Two grasslands (HD-IT) showed no difference in alpha diversity across the complexity gradient.

Two grasslands align with the general observed patterns in which there is a significant decrease in Min for both alpha diversity parameters compared to Max (BZ and GER). There are differing patterns per grassland, both for ASV richness and Shannon-Wiener Index. Two out of five grasslands show no difference at all for both parameters across the complexity gradient (IT and HD). Two grasslands showed significant increases in alpha diversity in Low compared to Max (GER and HW). Alpha diversity parameters showed similar results per grassland, but sometimes differed in their significance. For a more detailed overview, please refer to Appendix 5.

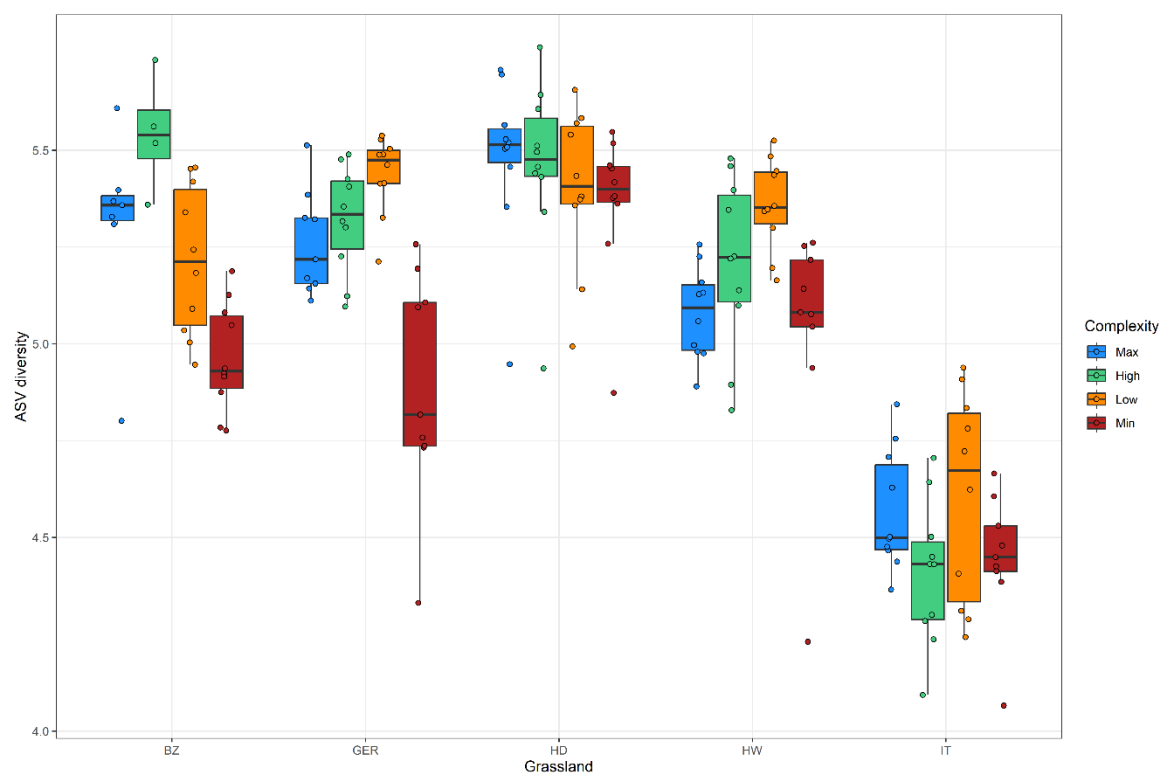


Figure 2. ASV diversity per complexity level per grassland. This figure shows the variation in ASV diversity, expressed as Shannon-Wiener diversity Index in each of the different grassland-complexity combinations. Note that the response of ASV diversity to complexity level differs per grassland.

Beta diversity

The dbRDA ordination shows that there are differences amongst the complexity levels across different grasslands (See Figure 3). PERMANOVA analysis confirms that there are indeed significant differences in bacterial communities between the complexity levels ($R^2 = 0.04632$ | $F_{3,183}=2.9628$; $p<0.0001$).

There is especially clear clustering of Min complexity, although not a singular cluster is formed. While this is likely in part an artefact of the ordination not being able to completely account for grassland variation (see Appendix 4), the addition of the NMDS ordinations reveals that the different Min clusters are mainly caused by differences in patterns across the grasslands (see Figure 4). Further, there is a modest clustering of Low which does overlap in part with High and Max.

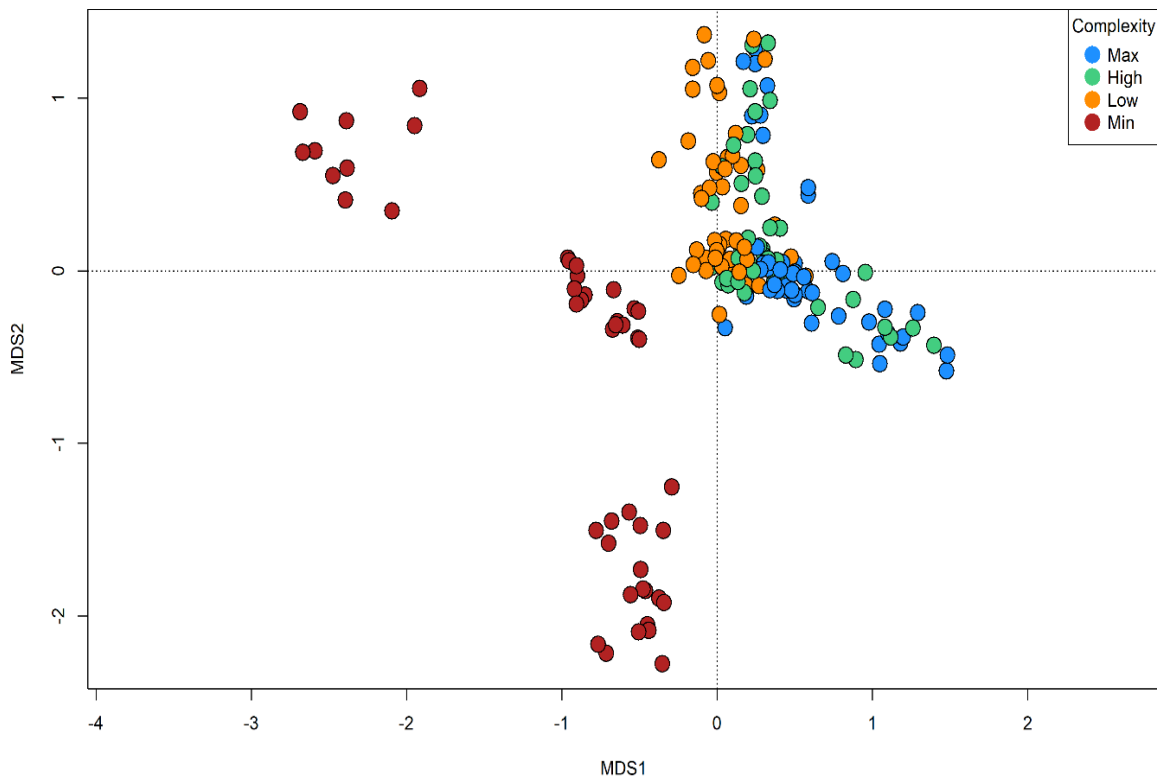
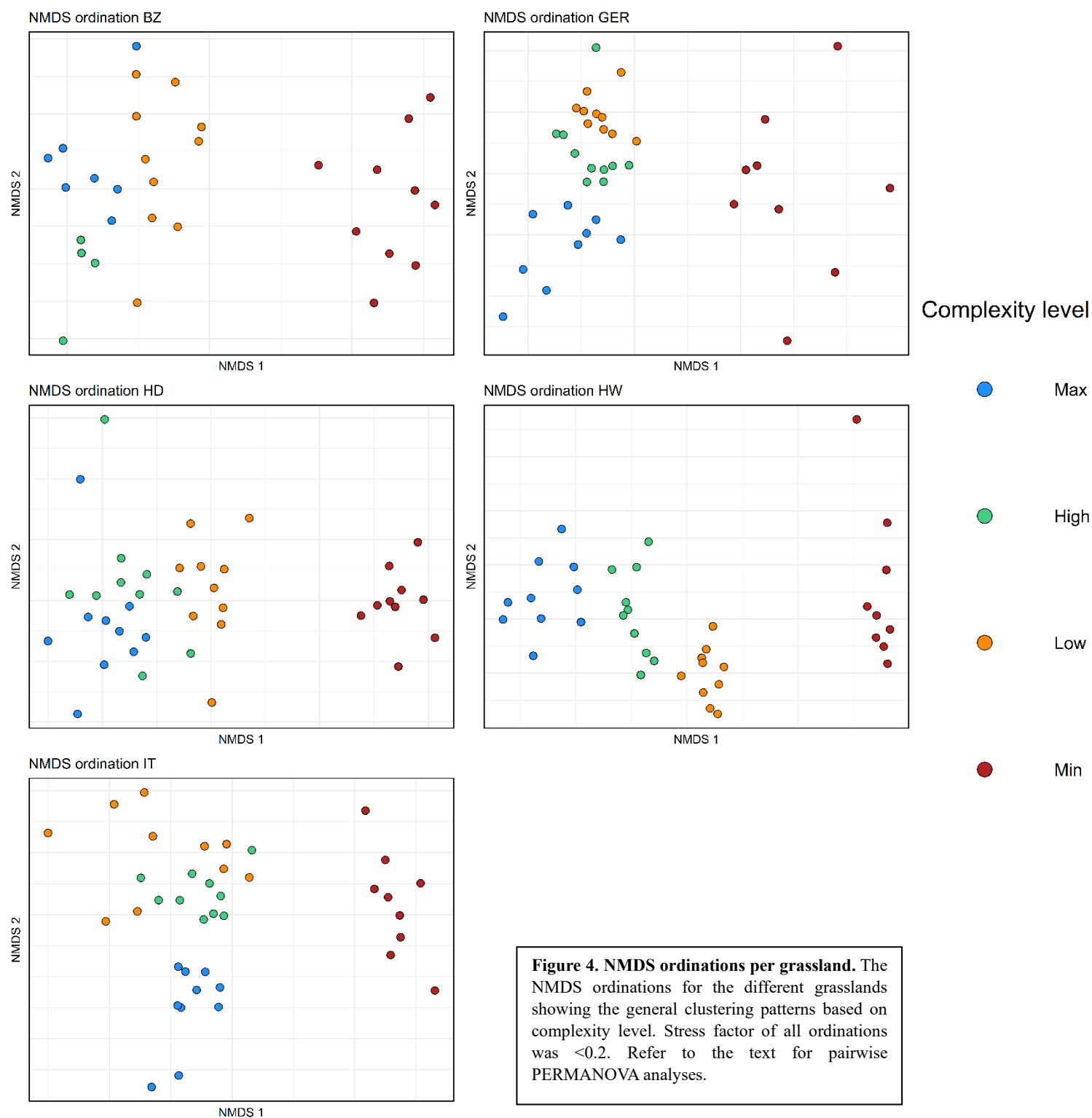


Figure 3. dbRDA of all samples. This figure shows the clustering of the different complexity levels in a distance-based RDA plot where grassland was used as a condition in order to more closely compare the communities based solely on Complexity level. MDS1 explains 8% of variation, while MDS2 explains 5% of variation. Refer to the text for results of PERMANOVA analysis, showing significant differences explained by complexity level.

In comparison with the dbRDA ordination, the specific NMDS ordinations more clearly show the differences between complexity levels across the grasslands. In all grasslands, the Min complexity is distantly clustered away from the other complexities, while complexity levels Max, High and Low are clearly more similar (See Figure 4). The pairwise PERMANOVA analyses confirm these visualised differences as all complexity levels are consistently significantly distinguished from each other across all sampled grasslands (See Appendix 6). Based on the R^2 values, we see that all five grasslands show a gradual gradient from Max to High to Low. There are differences in overlaps depending on the specific grassland, which is clearly visualised in the NMDS plots.

The complexity level most closely resembling the Min complexity is different depending on the grassland. Grasslands HD and HW continue the gradual Max-High-Low gradient as for these grasslands, Low is most similar to Min. This is clearly visualised in their respective ordinations. For grasslands BZ, GER and IT, the most similar complexity level to Min is High, with Max being the second most similar and Low being least similar. This pattern is not clearly visualised for BZ, which might be in part due to the low sample size of BZ High. Nevertheless, all pairwise comparisons with Min are highly significant.



Dominant phyla and genera

All ASV reads were identified on phylum level and 73.84% on genus level (see Table 1).

The 10 dominant phyla comprise ~96.5% of the entire dataset, with the remaining 30 phyla comprising only 3.5% (See Figure 5). Notable proportions get taken up by Firmicutes (30.4%), Proteobacteria (26.7%) and Actinobacteriota (14.3%), together representing 71,4% of all ASV reads.

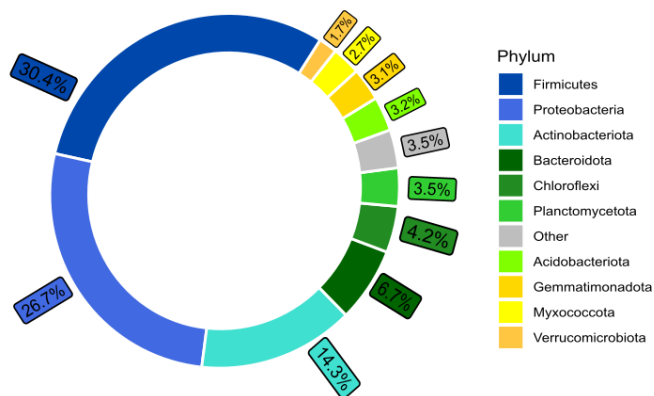
The 10 dominant genera comprise 42.1%, with the remaining 57.9% of reads belonging to 650 different genera which comprise at least 1 read (See Figure 5). The most notably abundant genus is *Bacillus*, which accounts for 22.6% of all ASV reads identified on genus level. The other 9 dominant phyla all have a similar representation (1-3%) within the dataset.

Nine out of ten dominant phyla show a significant difference in relative abundance in at least one of the complexity levels compared to Max (See Figure 6). The same can be said for 7 out of 10 dominant genera. Relative changes for dominant phyla were between -36% and 44% (adjusted p values in the range 0.02 - 2.4e-10), while those of dominant genera were between -40% and 143% (adjusted p values in the range 0.04 - 1.12e-17).

Table 1. Overview of Taxonomic assignment. This table summarises the total ASV reads in the ASV table and the percentages stating what proportion of these reads were assigned by the SILVA database on Phylum and Genus level. Note that ASVs which were not assigned on Kingdom level had already been filtered out during processing of the data in the Bioinformatics step.

<i>Total ASV reads</i>	<i>Total reads assigned on Phylum level (%)</i>	<i>Total reads assigned on Genus level (%)</i>
1,228,192	100	73,84

Dominant Phyla



Dominant Genera

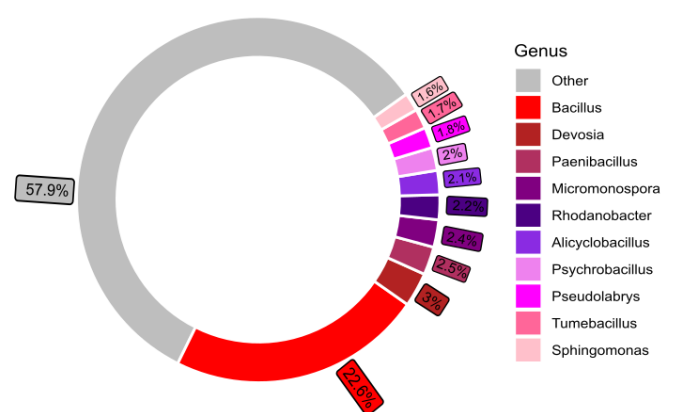
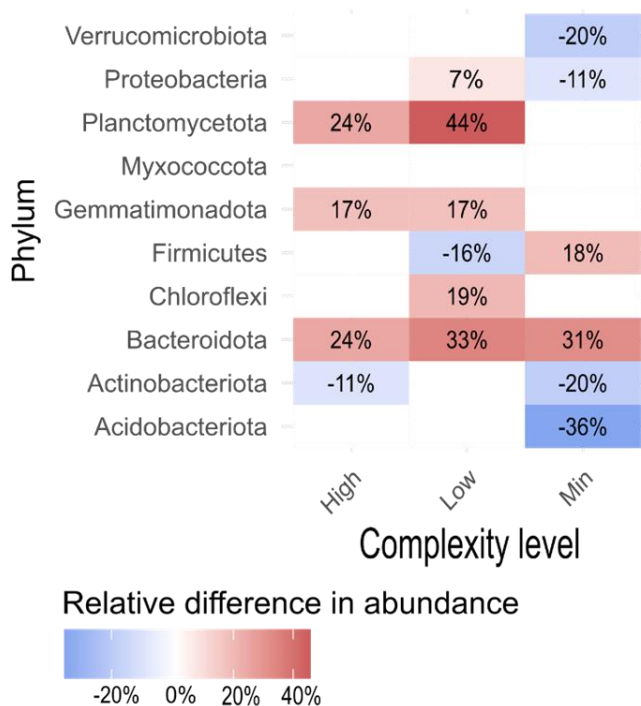


Figure 5. Representation of dominant taxa in the ASV table. Left shows what proportion of total ASV reads is represented by the 10 individual dominant phyla, with the 30 remaining phyla aggregated together in the “other” category. Right applies the same principle to the 10 dominant genera, but only considers the ASV reads which were assigned a genus (73.84%) . “Other” comprises 650 remaining genera.

Dominant phyla



Dominant genera

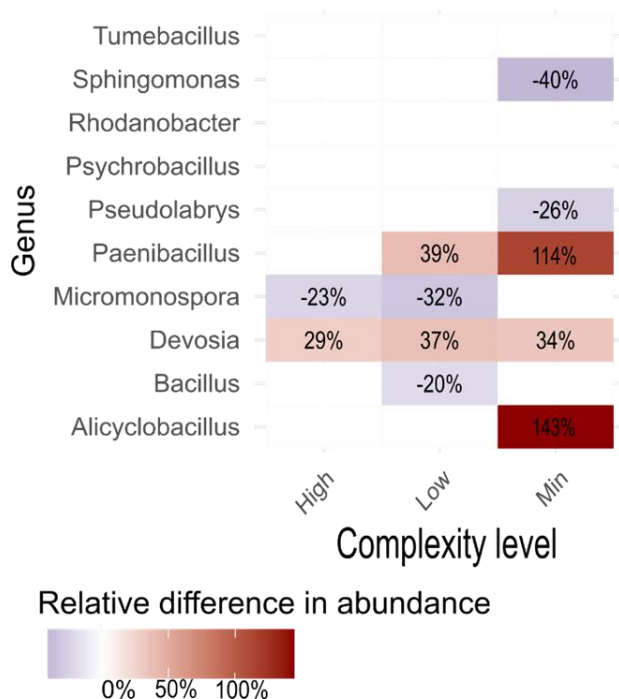


Figure 6. Relative changes in relative abundance of dominant taxa per complexity level. These heat maps show the significant relative changes of the mixed effect models for the dominant phyla (Left) and dominant genera (Right) compared to the relative abundance in Max. Red colouring indicates a significant increase compared to Max, while blue colouring indicates a decrease. Labels inside the cells show the exact proportional change. Note that different hues were used for the two heat maps to appropriately complement the different scales. For further details, see the text.

3.2 Nitrogen cycling

ASV representation

Out of the 9,915 unique ASVs across the dataset, ~30% of ASVs were assigned at least one general function through the FAPROTAX database (See Table 2). This is a comparable rate to other studies and deemed appropriate for further analysis¹¹⁵. Further, the table shows that a clear minority (~1,2%) of the total ASVs were identified to perform one of the N cycle functions. Additional analysis confirmed that not a single ASV carried out multiple of these functions. For all grasslands, nitrogen fixation is the function that has the highest ASV representation, with an exception for IT, as it has an equal number of ASVs for nitrogen fixation and nitrification. Out of the 27 total nitrification ASVs, 24 were found to belong to the Kingdom Archaea.

The generalised linear mixed effect model for nitrogen fixation shows that there are no significant differences in the number of ASVs across the complexity gradient. All of the grassland specific glms confirmed this pattern.

The glmer for the effect of complexity on nitrification shows that all reduced complexity levels contain significantly fewer ASVs compared to Max (High: $z=-3.551$; $p<0.0004$ | Low: $z=-5.683$; $p=1.32e-08$ | Min: $z=-8.027$; $p=9.97e-16$). A post-hoc emmeans test showed that all pair-wise comparisons (except for High-Low) were also significant, with a decreasing number of ASVs as complexity. All specific generalised linear models showed at least part of this pattern, with IT being the only grassland to also show a significant decrease in all reduced complexity levels compared to Max.

The model for denitrification showed that Min had significantly fewer ASVs compared to Max ($z=-0.0421$; $p=0.0421$), while both High and Low showed no significant differences in comparison. A post-hoc emmeans test showed no significant pair-wise comparisons however. For the specific Grasslands, only IT showed a significant decrease in ASV number, in Min ($z=-1.984$; $p<0.048$).

Table 2. ASV representation of N cycle functions in different complexity levels per grassland. (Top) This table summarises first the total number of unique ASVs across the dataset. Next, the assignment rate of the FAPROTAX database, indicating the percentage of total ASVs which were assigned at least one function of all functions FAPROTAX is able to assign. Out of the total ASVs, it shows how many were assigned the three different nitrogen cycling functions (which did not overlap). **(Bottom)** Further is shown what number of these N cycling ASVs were found in each grassland (yellow marking) and then gives the mean number of ASVs found per complexity level within that grassland (with standard deviation).

<i>Total unique ASVs</i>	<i>Assignment rate</i>	<i>Total N fixation ASVs</i>	<i>Total nitrification ASVs</i>	<i>Total denitrification ASVs</i>
9.915	29.40%	73	27	22
<i>Grassland</i>	<i>Complexity level</i>	<i>N fixation ASVs</i>	<i>Nitrification ASVs</i>	<i>Denitrification ASVs</i>
<i>BZ</i>	All	25	3	5
	Max	8.9±1.8	1±0.6	1.3±0.5
	High	10.3±2.2	1±0	1.3±0.5
	Low	7.7±1.9	0.4±0.5	1.1±0.3
	Min	6.8±1.6	0.1±0.3	1.2±0.4
<i>GER</i>	All	35	5	10
	Max	7.2±3	1.1±0.3	1.8±1.2
	High	6.4±2	0.4±0.5	1.8±0.9
	Low	6.2±1.8	0.1±0.3	2.7±0.7
	Min	5±1	0.3±0.5	1±0.5
<i>HD</i>	All	42	17	15
	Max	6.2±3.3	6.1±2.1	1.6±1
	High	7.6±2.4	4.7±2.3	1.7±1.1
	Low	5.6±1.6	2.6±1.5	1±0.9
	Min	6.8±2.3	1.5±0.5	0.7±0.8
<i>HW</i>	All	18	8	3
	Max	2.9±0.9	4±1.2	0±0
	High	4.1±1.6	2.9±1.3	0.2±0.4
	Low	3.3±1.3	3.2±1.3	0.5±0.5
	Min	3.1±1.7	1.4±0.5	0.5±0.5
<i>IT</i>	All	9	9	3
	Max	4±0.7	5.5±0.7	1.2±0.6
	High	3.1±0.7	2.7±0.9	0.7±0.7
	Low	3.4±0.5	2±1	0.6±1.1
	Min	3.9±0.6	1±1.1	0.3±0.5

Potential functioning

Nitrification and denitrification were both affected by soil biotic complexity, while nitrogen fixation was not (see Figure 7). Patterns differed in magnitude for different grasslands (see Figure 8).

Nitrogen Fixation

There were no significant differences in the relative abundance of nitrogen fixers. Not in the mixed effects model, nor in the separate linear models per grassland.

Nitrification

Relative abundance of nitrifying bacteria was decreased in High by ~60% ($t = -6.612526$; $p=0$), in Low by ~66% ($t = -7.913302$; $p=0$) and in Min by 98% ($t = -10.1098662$; $p=0$) compared to Max (intercept: ~0.72% relative abundance; $p=0$) in the mixed effect model. The Tukey post-hoc test additionally revealed that Min has a significantly decreased relative abundance compared to High ($z = -3.322$; $p = 0.00494$), while other pair-wise comparisons were not significant. For the specific linear models: BZ showed the general pattern of the mixed effect model. For HD, HW and IT, relative abundance of nitrifying bacteria was significantly decreased in all of the reduced complexity levels compared to Max. For GER, High and Low had significantly decreased relative abundances compared to Max .

Across the dataset, 4,305 reads belonged to one of the 27 nitrifying ASVs. The 24 archaeal ASVs accounted for 4,011 of these reads (~93%). This indicates the relative importance of Archaeal taxa for potential nitrification as estimated here (See Appendix 10).

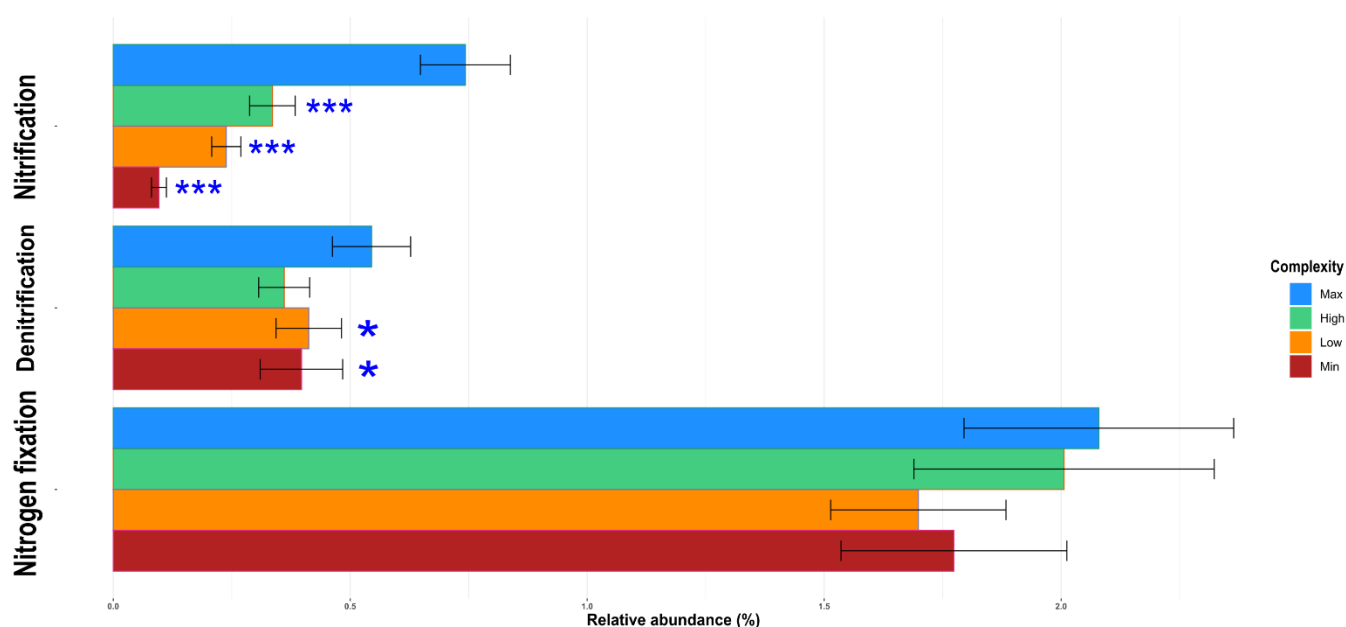


Figure 7. Relative abundance of the N cycle functions per complexity level. This bar plot shows per complexity level the mean relative abundance of ASV reads which were assigned the functions nitrification, denitrification and nitrogen fixation. Standard deviations are given. Significance indicators from the mixed effect models are shown in blue to indicate a significant difference compared to Max. This bar plot was created using the microeco package. Grassland variation was not taken into account, but was in the model.

Denitrification

The mixed effect model shows that the relative abundance of denitrifying bacteria was significantly lower in Low ($t = -2.549395$; $p = 0.0116$) and Min ($t = -2.881569$; $p = 0.0044$) compared to Max. The post-hoc Tukey test reveals no further significant differences. Specific linear models showed either results that were in line with this general pattern (BZ and IT), no differences across the complexity gradient (HD and GER) or in the case of HW, an increased relative abundance in Low.

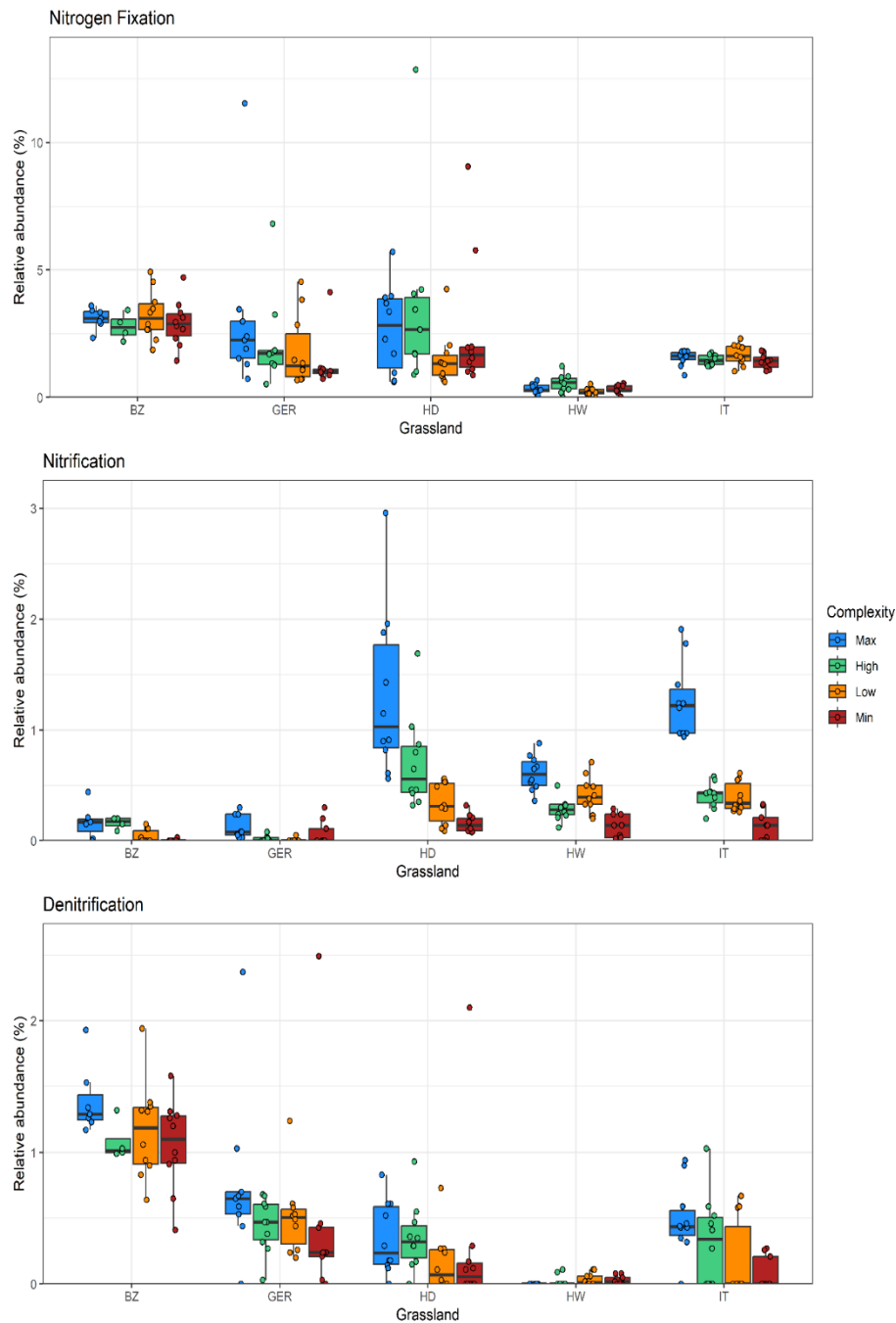


Figure 8. Relative abundance of the N cycle functions per grassland per complexity level. These box plots show the variation of the different N cycling functions across the grasslands. Note that the three plots have different scales for the y axes.

3.3 Plant Productivity

For the case studies, some interesting cases will be described here. For the full description, refer to Appendix section “supplement model outputs”.

The linear mixed effect models show that there are no significant differences in general for aboveground biomass, neither were there differences in grass biomass. This was reflected in the linear models of all grasslands, except IT, which had a significant decrease of AGB compared to Max in High ($t_{36}=-2.260$; $p=0.03$).

LMM for Litter (with normality of residuals corrected with square root transformation) was significantly decreased by ~ 0.19 g ($\sim 19\%$) in Min ($t_{192}=-2.174$; $p<0.03$). A post-hoc Tukey test found no significant differences for any pair-wise comparison. Most remarkable was grassland IT, which has a significant decrease in litter of $\sim 20\%$ in all reduced complexity levels (range: $t_{36}=[-2.76;-3.23]$; $p<[0.009;0.0027]$). A supplementary Post-Hoc Tukey test revealed no further differences.

Since aboveground biomass and grass biomass did not change (with one exception), we will omit the tests for herb and root biomass and directly show the test for herb-grass ratio and root-shoot. See Appendix 15 for the results of the separate biomass parameters.

LMM for Herbs:Grass biomass ratio (with normality of residuals corrected with log transformation) was significantly decreased by ~ 0.03 ($\sim 33\%$) in High ($t_{192}=-2.397$; $p<0.018$) and by ~ 0.06 ($\sim 60\%$) ($t_{192}=-5.55$; $p<0.00001$) compared to Max. A post-hoc Tukey test showed the ratio in Min was significantly decreased compared to all other complexity levels (range: $p<[0.08;0.001]$), with no further significant pair-wise comparisons.

Remarkably, Herb:Grass biomass ratio in HW compared to Max was decreased by ~ 0.12 ($\sim 50\%$) in High and Low, with a more pronounced decrease ~ 0.18 ($\sim 75\%$) in Min (range: $t_{36}=[-5.46;-8.168]$; resp. $p<3.13e-06$; $p<3.68e-06$; $p<1.03e-09$). A supplementary post-hoc Tukey test for this grassland indicated that there was a significant decrease in Min compared to Low ($p<0.04$), but not compared to High.

Root:shoot ratio is significantly increased by 0.04 ($\sim 10\%$) in Low [$t_{192}=1.99$; $p<0.048$] and by 0.05 ($\sim 12\%$) in Min [$t_{192}=2.49$; $p<0.014$] compared to Max. Post-Hoc Tukey test did not find any significant differences.

The significant results of the LMMs are presented in Figure 9.

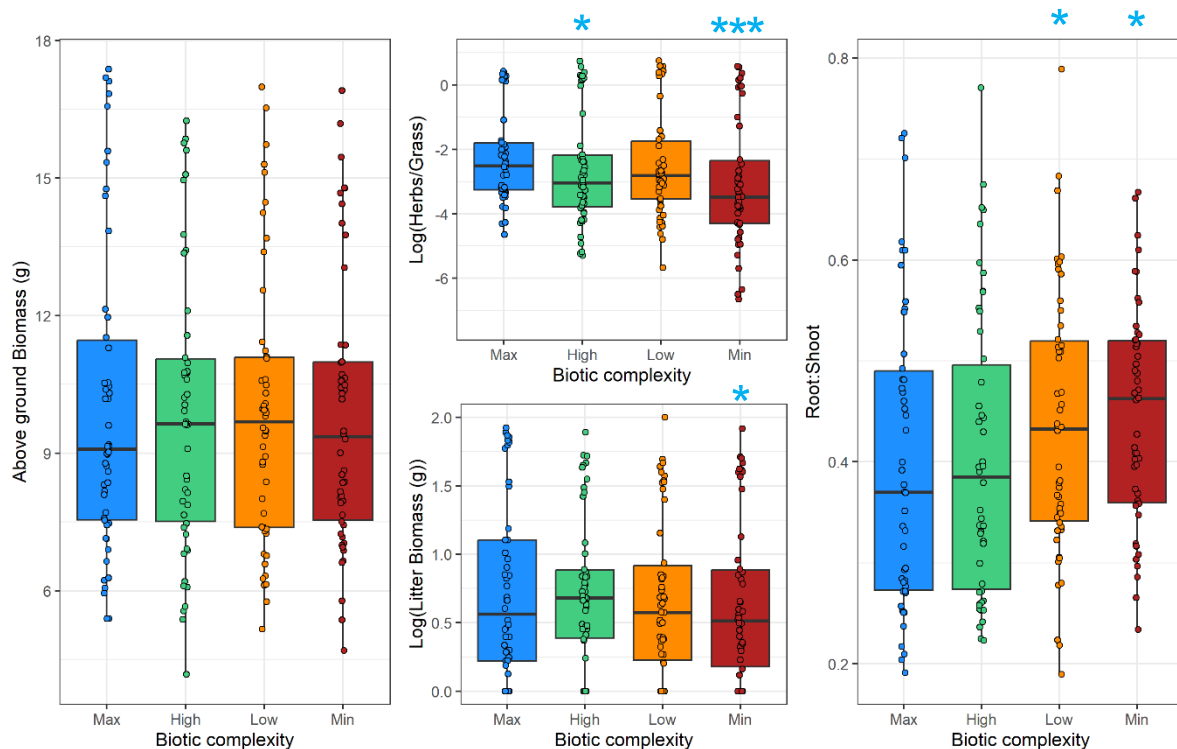


Figure 9. Biomass parameters across the complexity gradient. Left shows the variation in aboveground biomass per Complexity Level. **Middle (top)** shows the variation in proportion of Herb to Grass biomass. For a better visualisation, the proportion was log transformed. One datapoint ($y=-10$) was omitted for Min to improve visualisation further. **Middle (bottom)** shows variation of litter biomass. The parameter was $\log_{1p}$ transformed to improve visualisation, while correcting for microcosms which did not contain any litter. **Right** shows Root-Shoot ratio across the complexity gradient.

Significance indicators based on the respective LMM models were added (see text). These were coloured blue to highlight comparison with Max.

4. DISCUSSION

In this thesis, we investigated the influence of soil biotic complexity on bacterial community composition, the efficiency of bacterial nitrogen cycling and plant productivity. Bacterial communities were supplemented with Archaeal data to give a broader overview of nitrification. We were interested in the interactions in these soil communities and the effect of eliminating them from the system. Our results underline the importance of a well established soil organism network as all three studied facets were impacted by biotic complexity. Not only the importance of complexity in general was underlined, but also the influence of specific taxonomic groups as no two treatments had identical responses for all facets of the study.

We proposed that the driving forces of potential differences could be linked to trophic interactions (top-down regulation), competitive interactions, niche opportunities, the decomposition network, the microbial loop and functional redundancy.

4.1 Bacterial communities

Alpha Diversity

Alpha diversity, both ASV richness and diversity (Shannon-Wiener Index), in the mixed effect model declines only in the most reduced complexity level, Min. This is in line with our hypothesis, although we suspected a more gradual decrease with decreasing biotic complexity. Fungi and protists thus increase bacterial diversity, presumably by exerting an increased competitive and predatory pressure respectively on otherwise dominating bacterial taxa^{55,69}. Although new niches should also be present, benefiting for instance fungivorous bacteria and bacterial pathogens^{69,116}. Bacterial communities in Max, High and Low all are characterised by a higher alpha diversity (at least in one of the parameters) than Min, with no further differences between them. This presumably indicates the contrasting conditions between Min, which lacks interactions with other taxonomic groups, and the other complexity levels, where phylogenetically different groups interact with bacteria. Fungi+Protists, Microfauna and Mesofauna can each impact bacterial diversity, which is evident in the results of the case studies.

As expected, specific grasslands show a range of different patterns. While GER matched the general pattern, alpha diversity in IT and HW was not impacted by soil biotic complexity. Grassland HW had the most diverse bacterial communities in Low, while BZ in high. This wide range in specific patterns very clearly showcases that different biotic (e.g. plant community composition and different representation of functional groups) and abiotic conditions (e.g. soil fertility levels and pH) can co-regulate the response of bacterial communities to soil biotic complexity. Post-hoc Tukey tests are likely to describe the intricate patterns more clearly and should be considered in the continuation of this research.

A general consensus view is that soil bacterial communities are shaped by bottom-up control ^{117,118}. Differences in soil fertility are therefore partly responsible for the specific dynamics in grasslands. However, our results show that top-down control is an important regulatory mechanism as well, which is especially clear from the non-linear patterns in HW and BZ. This has also been shown in a study by Asiloglu et al. (2021) for instance, where top-down regulation of bacterial grazing protists outweighed bottom-up effects ⁵⁵. Further, Romdhane et al. (2022) found in their experiment that 39% of dominant bacteria were subjected to competition during recolonisation events and that the absence of certain microbial taxa influenced the success of bacterial taxa differently ¹¹⁹.

Differing patterns in alpha diversity could possibly be explained by a total bacterial grazing pressure exerted by all different soil taxa, which can either increase with complexity when more bacterial grazers are present or decrease as secondary predators are present. Different nematode taxa can for instance be bacterial grazers or protist predators.

Beta diversity

Our hypothesis stated that bacterial community composition is heavily influenced by the level of complexity of the soil community they are part of, with clear differences between all treatments. Our results show that this is true for all studied grasslands, although to a differing degree. Even both grasslands which did not alter in their alpha diversity did still show significant differences in community composition between treatments.

Particularly interesting is the gradual gradient from Max to High to Low which is seen in all grasslands. It indicates that the taxonomic groups, which are sequentially represented in the soil communities, all have their own influence on the bacterial communities. This was what we expected since the interaction networks in soils make it so organisms are dependent on each other. Our results show that the effects clearly ripple throughout the soil network. This highlights our belief that researchers should strive more to capture different aspects of the soil biotic world in their experiments.

There are two main patterns when it comes to comparing Min with other complexity levels. Either, the complexity gradient continues, with Min sharing the most similarity with Low (HD and HW). Otherwise, Min shares most similarity with High, then Max and the least with Low (BZ, GER, IT) (See figure 4).

I argue that both patterns can be explained in part through the intensity of predative/competitive pressure. The Max-Min gradient aligns with my hypothesis and thus it is possible that for these grasslands, niche opportunity, predation and competitive pressure rise gradually with an increasing soil biotic complexity. The effect of this would then express itself in “pushing” the bacterial communities in a certain direction. Another possibility could be that bacterial communities are changing in function of decomposition efficiency. If these bacterial communities are highly dependent on the decomposition

performed by these larger organisms, then disturbance in our illustrated conveyor belt, could stunt bacterial performance and drive them in another direction.

The highest similarity of bacterial communities in Min with those in High followed by Max could indicate that the selective predative and/or competitive pressure on bacterial communities is highest in Low (which contains protists and fungi), suppressing the bacterial taxa which are dominant in Min. These pressures could then gradually be alleviated as more complex organisms, such as nematodes and collembola, affect the performance of these competitors and bacterial grazers. For example, both fungi and bacterial grazing protists are known to be affected by predatory nematodes^{58,120}.

Dominant bacterial taxa

Dominant phyla

Community changes are quite pronounced as they can be detected even at Phylum level and for a wide variety of phyla. Nine out of ten dominant phyla have a significantly different relative abundance in at least one of the reduced complexity levels compared to Max (see Figure 6). These results show that changes in the complexity of the soil system have large implications for the bacterial community composition and thus possibly for ecosystem functioning as well.

Some patterns in our experiment were found to align with pre-existing literature, such as the decline of Acidobacteriota in systems with low predation pressure, but other patterns seem to contradict literature, such as Proteobacteria decreasing under conditions with low predation pressure (Min) and increasing when bacterial grazers are present (Low)^{54,119,121}. It is worth mentioning that many studies on top-down regulation in soil communities often regard a simple experimental system which can sometimes contain just one bacterial grazer species. While this shows a clear dynamic for the studied species, soil networks are far more complex, which will alter these dynamics at least in magnitude. This could be the reason why our result for Proteobacteria differs from these studies.

Dominant genera

Soil biotic complexity further expresses itself in the relative abundance of genera. Seven out of ten dominant genera were impacted by the complexity gradient. We will briefly explore further what these significant shifts could mean for ecosystem functioning.

In less complex soil communities compared to Max, we found that the relative abundance of *Devosia*, *Paenibacillus* and *Alicyclobacillus* increases with nuances depending on the genus. They could thus perform their respective functions at a higher rate in less complex systems. *Alicyclobacillus* can cause large-scale fruit spoilage and can form a problem in agriculture, especially in orchards¹²². Considering the wide use of fertilisers and pesticides decreases biodiversity, *Alicyclobacillus* could potentially thrive in these circumstances as its relative abundance is more than doubled, with possible implications for economic losses and food shortage. However, both *Devosia* and *Paenibacillus* are suggested to fix

nitrogen, while the latter performs other functions as well, such as phytohormone production and phosphate solubilisation ^{123,124}. Both could thus benefit plant productivity in less complex systems, potentially serving as a buffer when multifunctionality declines.

Conversely, the relative abundance of four of the other dominant genera declines in certain reduced complexity levels compared to Max. *Micromonospora*, *Pseudolabrys*, *Sphingomonas* and *Bacillus* all can contribute to plant performance, each through different processes, such as improving root ecology, nitrogen fixation, phytohormone production, bioremediation of contaminants and nitrification ^{125–128}. The decrease in the relative abundances of these genera can thus be problematic for the dynamics within ecosystems, especially since they appear to be amongst the most dominant genera in grasslands.

Especially the relative abundance decrease of *Bacillus* could negatively affect plant performance as it was by far the most ubiquitous genus in our ASV table (~16% of all ASV reads and ~22% of all reads assigned on genus level) (See Appendix 7 for the relative abundance of *Bacillus* per sample). The review paper of Saxena et al. (2020) summarised the varied array of processes different *Bacillus* species perform, including nitrogen fixation, mineralisation of phosphorus, phytohormone production, production of antimicrobial compounds and inducing systemic resistance to abiotic stress ¹²⁸. Recognising the potential of *Bacillus*, this thesis attempted to research a possible effect on plant productivity through mixed effect models, but no significant differences were found. However, *Bacillus* is also a key player in increasing soil fertility. Radujković et al. (2023) has found that the relative abundance of *Bacillus* was significantly higher in grassland sites that had higher levels of productivity ¹²⁹. Our data seems to align with this study, as the grassland microcosms that produced the most aboveground biomass generally had a higher relative abundance of *Bacillus* (See Appendices 7 and 9) [Note that grasslands HW, GER and IT were sampled in their study as well, although data collection was independent]. Moreover, the grasslands with the highest biomass production, and thus the highest relative abundance of *Bacillus*, were reported by local managers to be the most productive in comparison (see Table A). If there is indeed a causal relationship, then the decreased relative abundance of *Bacillus* by reduced soil biotic complexity (in complexity level Low) could have grave implications for plant performance and soil fertility. This would make *Bacillus* a primary microbial conservation target in the fight against global change. Further research is therefore advised.

In essence, our findings show us that the majority of dominant genera are impacted in terms of their ubiquity by a gradient of biotic complexity. In most cases, the decrease or increase of these genera due to a decrease in biotic complexity coincides with a potentially lower rate of ecosystem functioning or higher rate of possible health endangerment of both humans and ecosystems. In some cases however, the relative abundance shifts could theoretically be beneficial for these genera and by extension, the functions they perform and the ecosystems that benefit from them. It is important to keep the larger

network of dynamics into account to determine if reduced biotic complexity would in general be beneficial, harmful or neutral for ecosystems.

4.2 Nitrogen cycling

In general, the efficiency of nitrogen cycling as performed by bacteria was clearly impacted by a reduced soil biotic complexity.

Nitrogen Fixation

Out of the three studied functions, only nitrogen fixation showed no general shifts in relative abundance or ASV representation in function of biotic complexity.

We propose two concepts as an explanation for this seemingly balanced state of potential nitrogen fixation across the complexity gradient. First, nitrogen fixers don't exclusively depend on decomposition to sustain their nutritional needs as they fix nitrogen from the atmosphere and often are in close contact with plants to retrieve carbohydrates – through symbiosis via root nodules or in the rhizosphere feeding on exudates (free-living nitrogen fixers) ^{98,99,130}. Since plants were present in all complexity levels, nitrogen fixers would in theory have had access to carbon across the complexity gradient. This partial decoupling of their dependency on the soil network could allow nitrogen fixers to keep their performance balanced in situations where complexity has decreased. Secondly, ASV representation is comparatively much higher for nitrogen fixation in all grasslands than for the other two functions (with IT having the same number of ASVs for nitrogen fixation and nitrification). We also see that in most grassland the complete set of nitrogen fixers is much larger than the mean number of ASVs per complexity level. There is thus a relatively high level of functional redundancy, while these ASVs don't seem to co-exist in large numbers relative to the “complete set”. I propose that this highlights the importance of functional redundancy ⁴⁴. It is plausible that each nitrogen fixer of the overall community of the donor soil has a different set of environmental requirements. This would mean that they occupy different niches, as prime example, niches which are the result of different levels of biotic complexity and the accompanied implications. In order to keep the relative abundance of nitrogen fixers stable, different ASVs would have to show different patterns across the complexity gradient, which should more or less be complementary to each other. In analysis not displayed in full in this thesis, the relative abundance of a few potential nitrogen fixing ASVs and genera were studied. Indeed, nitrogen fixers as a group did not have a uniform relationship of relative abundance across the complexity gradient. Before, we already discussed the genus *Devosia* and its increased relative abundance in reduced complexity levels. Conversely, the relative abundance of *Pseudolabrys* decreased in Min. Another genus we looked into further was *Bradyrhizobium*, also a root-nodulating genus, which showed the opposite pattern in the sense that it had its highest relative abundance – across four out of five grasslands – in complexity level Max (See appendix 11).

An important note however is that nitrogen fixers often fix nitrogen facultatively, meaning they only perform the function under certain conditions ¹³¹. It can therefore not accurately be concluded that a higher relative abundance of nitrogen fixers also translates into a higher fixation rate of nitrogen. A large part of root-nodulating symbionts for instance only perform the function when in symbiosis with their plant host. However, in contrast to the other two functions, the potential to fix nitrogen remains in tact.

Nitrification

For nitrification, it is very evident that a lower complexity level translates both into a lower relative abundance of nitrifying bacteria, as well as a less diverse representation of ASVs. While we argued before that the relative abundance is not always a suitable proxy for the rate of a function, the relative abundance of nitrifiers has been shown to be an adequate proxy for the nitrification rate ⁸². Our results thus strongly suggest that nitrification rates strongly decline – across multiple studied grasslands – concomitantly with soil biotic complexity. This means that the different soil organism groups – protists/fungi, microfauna and mesofauna – indirectly influence (prokaryotic) nitrification rates. Again, all these taxonomic groups contribute to decomposition, which would increase availability of ammonium, increasing the substrate for nitrifiers. Top-down regulation could in theory also alleviate potential predation pressure on nitrifiers, but related studies have found differing responses of nitrification rates to predation pressure ^{132–135}.

Especially surprising was that the vast majority of nitrifying ASVs were Archaeal rather than Bacterial. Closer inspection of potential nitrification then revealed that it too was dominated by Archaeal ASVs (See Appendix 10). This was an unexpected find as Archaea only made up 0.33% of the ASV table, distributed over just 32 ASVs (out of 9.915 total). Although Archaea also dominated as nitrifiers in other studies ^{92,135}. BZ was the only grassland that exclusively showed bacterial nitrification. Interestingly, in GER and HD, the pattern of potential Archaeal nitrification suggests that Archaea are even more strongly declining than potential general nitrification. It seems even that Archaea are completely absent in these Min complexity levels, while we still see some potential (bacterial) nitrification. This could indicate some form of niche differentiation or competitive exclusion of nitrifying Bacteria by Archaea ^{136,137}.

Previous studies have pointed out that the primer pairs used in this experiment (515F/806R) fail to capture Archaeal DNA in extractions at high efficiency, notably DNA of the class Crenarchaeota, which contains nitrifying Archaea (although there were ASVs in the dataset that belonged to this class and were considered nitrifiers) ^{135,138}. Since the most prominent nitrifiers in our results are Archaea, it could mean that potential nitrification is underestimated. If this is the case, then the decreasing pattern for nitrification along the complexity gradient we found might be even more pronounced in reality than in the observed pattern.

Denitrification

The relative abundance of denitrifying bacteria and the representation in number of ASVs decreases with a decreasing complexity. Denitrification is both linked to the decomposition process and nitrification process – both providing nitrogen compounds which are suitable substrates for denitrification. A similar dynamic might therefore be present for denitrifying bacteria as we explained for nitrifiers with the direct and indirect dependency on the decomposition process ⁹⁴.

Since both nitrification and denitrification decline with a decreasing biotic complexity, relative outflux of nitrogen from the system might not differ compared to more complex systems. This might be a ‘benefit’ for scenarios in which nitrogen is limited, decreasing the abundance of nitrates leaving the system for instance. However, in cases of nitrogen deposition this could become a detriment as denitrifying bacteria could help to alleviate the excessive nitrogen load from the polluted ecosystems. Denitrification has been valued in other studies to aid in this problem not only in natural conditions, but also in agriculture ^{139,140}.

Both nitrifying and denitrifying bacteria have a much lower level of ASV representation compared to nitrogen fixation in our results, which could further explain the decrease in relative abundance for these functions as there is less of a safety net in place to remain active in the system ⁴⁴. This is for instance especially clear for grassland IT, which has a total denitrification representation of just three ASVs across the complexity gradient. We see that with decreasing complexity, a higher proportion of microcosms was totally void of reads (see Figure 8). This can be due to specific microcosm circumstances or just stochastic events which prevented any of the ASV representatives from establishing. It thus becomes even more imperative that functional redundancy – the presence of diverse bacterial taxa performing the same function – has an important role in the ecosystem when it comes to resilience to varying conditions ^{38,40,44}.

Our results clearly show that nutrient cycling in less complex soil systems, besides being limited by a less intricate decomposition network, is further limited as prokaryotic nitrifiers and denitrifiers have declined in relative abundance. The significant decrease in potential nitrification, observed consistently across all grasslands (either in total or archaeal nitrification), is a particularly important finding. If less ammonium is being converted into nitrates, then plants will have to mostly rely on the prior, which is toxic in high conditions ⁹¹. Plant productivity, and perhaps the performance of other organisms as well, is likely to decrease in these systems in the long run, which will have implications for the dependent biota.

4.3 Plant Productivity

Aboveground biomass

Contrasting with our hypothesis, the results show that grass biomass was unaffected across the complexity gradient. Grass biomass made up the largest proportion of aboveground biomass which would explain the similar pattern for AGB. However, the biomass of herbs did change across the complexity gradient, although not in a linear way as our hypothesis suggested. Wagg et al (2014) found a similar discrepancy in the response of grasses versus herbs^{112,141,142}. Interestingly, herb biomass is most decreased in Min, not impacted in Low and decreased in High compared to Max. This potentially suggests (1) that especially the absence of fungi and/or protists (present in Max-High-Low) is detrimental for herbs, while (2) microfauna (Max-High) have an antagonistic effect on herb productivity, which (3) is compensated for by mesofauna (Max). A possible explanation is that (1) increased resource take-up provided by mycorrhizal fungi and/or mineralisation due to protist bacterial grazing increases the performance of herbs, which is in turn (2) partly negated by fungivorous / protozoan grazing / phytophagous nematodes which either decrease the prior benefits or directly put stress on herbs^{58,67,143}. (3) These trophic interactions could then be suppressed via top-down regulation by collembola or acari or their general plant growth promoting effects^{53,58,63,65}. While grasses are exposed to these interactions as well, herbs for instance are more dependent on their symbionts^{144–146}. Wang et al (2019) showed in their experiment that microbes, notably mycorrhizae, promote plant growth of forbs disproportionately compared to grasses and that plant pathogenic nematodes reduce plant growth of some forb species, but also of some grass species¹⁴⁷. Although Eisenhauer et al (2010) found that nematodes especially decrease the shoot of non-legume herbs (which the majority of the herbs in this experiment were)¹⁴⁸.

Among the grasslands, especially HW was interesting. It showed highly significant differences in the ratio of Herb:Grass biomass in all reduced complexity levels compared to Max, which was the most pronounced in Min. In this grassland, it thus seems that the beneficial effects on herb biomass of fungi and protists is not reduced by microfauna and is only further enhanced by mesofauna. Potential drivers aside, it can be concluded that herb productivity in this grassland decreases almost linearly with a decreasing soil biotic complexity. Since this directly reflects the competitive ability of herbs, plant communities in this grassland could become less diverse as their survival and reproduction rates decline under these reduced conditions. In fact, there already seems an indication of this. The encompassing experiment weighed the biomass of individual plant species per microcosm. The analysis of this data will be carried out in future steps of the research, but already we can see that herb species richness declined in this grassland, while grass species richness was equal across the complexity gradient (see Appendix 14). This gradual pattern of decline in herb species richness with decreasing soil biotic complexity matches that of herb biomass. However, there seems to be an indication that the germination rate and/or seedling survival was impacted in function of soil biotic complexity. Multiple studies have

shown the significant effect soil microbes have on these factors ^{149–152}. Further analysis of this data is thus encouraged to explore these dynamics further.

Most surprising was the fact that litter biomass was significantly lower in complexity level Min compared to Max. The most interesting case study was IT, which showed a significant decrease in litter of an even magnitude in all reduced complexity levels compared to Max. The litter variable might reveal more about the potential productivity of the plant community. It is possible that soil communities in Max promoted plant productivity in initial growth periods, perhaps primarily driven by the high potential nitrification (see Figure 8). The vegetation in these microcosms could eventually have reached a threshold in which the vigour of the vegetation could no longer be supported by the environment. This threshold could have been a deficit in nutrients or available light, as the vegetation in these microcosms was especially dense. Due to increased competition between the plants, relatively more plants would have died off compared to the more reduced complexity levels. This would explain why living biomass levelled off, while litter increased. Aside from acting as an indicator of potential productivity, we can also take the benefits of litter into account. For instance, accumulation of litter can provide seedlings with a more suitable environment, which helps them establish. In this regard, the production of litter itself could be seen as an ecosystem function, which in this study would be positively correlated with a more complex soil community ¹⁵³.

Root biomass

In the general pattern, Low and Min have an increase of ~10% root biomass compared to the Max complexity. This seems to be in line with the proposed hypothesis which stated that root biomass would increase when soil biotic complexity decreases as a stress response to nutrient limitation. This limitation would be the result of a degraded decomposition network and a degraded trophic network, which would both imply nutrient immobilisation, respectively in dead organic material and living biomass (highly competitive bacteria for instance). This hypothesis finds support in the results of potential nitrification, which decreased in all reduced complexity levels and also saw a negative correlation with root-shoot ratio (see Figure 8 and Appendix 12). If nitrogen is not readily available in a high abundance of nitrates, then plants would have to expand their roots in order to fulfil their nutritional needs. Further, nematodes are present in complexity level High and Max, expected to contribute to mineralisation, which would increase nutrient availability in these scenarios.

This response did not entirely align with our hypothesis, since it expected specifically the increase of root biomass when fungi – mycorrhizae – were absent as the reach of root networks would be considerably declined. The fact that root biomass increased in complexity level Low in a similar magnitude, suggests that the treatment had no differing effect on roots compared to Min. If this additional root growth was indeed due to the effect of nutrient limitations, mycorrhizae seemingly did not alleviate this stress. There is however, no direct evidence in literature that found mycorrhizae

reduced root growth. If anything, root-shoot ratio was found to decrease due to a higher production of aboveground biomass, but also in terms of root biomass, mycorrhizae were more inclined to have growth promoting effects rather than inhibiting growth^{101,154}. Further, while nitrification rates did indeed decrease with decreasing complexity, there was no significant difference between High and Low, possibly suggesting nitrification did not affect root growth in this experiment. This would leave the increased mineralisation rates by nematodes (Max-High) as the most plausible driver of decreased root growth in more complex soil systems, possibly in interaction with the prior two concepts.

The proposed hypothesis was only one possible explanation of many. Perhaps promotion of root growth in less complex systems was not a stress response at all. Considering there is no significant difference between Max and High, it could be the case that the presence of microfauna causes a direct reduction in root biomass in more complex systems. For instance, phytophagous nematodes reduced root biomass in a study of Verschoor (2002) by up to 40%¹⁵⁵. This would coincide with our previously proposed interaction, where phytophagous nematodes could be responsible for a decreased herb biomass in High. Since most roots belonged to grasses, indicated by the high relative proportion of aboveground biomass of grass, it would seem that phytophagous nematodes did feed on grass roots, but that they were not impacted in their aboveground productivity. It could be argued that the constant grass biomass is evidence of an increased relative productivity of grasses as they were able to produce similar amounts of aboveground biomass compared to complexity levels in which these nematodes were absent. This would again show the relatively superior resilience of grasses to stress compared to herbs. However, this whole reasoning remains speculative with the data provided in this thesis. Further analysis of the DNA extractions to analyse nematode communities could provide clarity on this topic.

Mesofauna had no noticeable effect on root biomass, which was against our hypothesis which expected a decrease in High compared to Max.

4.4 Considering the effect of methodology rather than treatment

The preparation and inoculation process differed slightly for the different complexity levels as a result of the filtering process being based on size. As mentioned before, the fine mesh size used to create inocula for the Min treatment proved to be somewhat of a hurdle. It prohibited us from supplying microcosms with the standardised 250 g of donor soil with the inoculum in the initial step, which was later compensated for with an additional inoculation step. This also meant that finer mesh sizes filtered coarser grain sizes out of the inoculum, although Wagg et al (2014) found that, in a similar experimental setup, overall soil properties of microcosms were not significantly impacted¹¹². A differing set of mesh sizes might have had a further impact as sieving methods can in fact influence bacterial diversity and abundance, although wet sieving (which more closely resembles the method in this experiment) is regarded to have a higher efficiency in comparison to dry sieving^{156,157}. Nevertheless, we cannot assume that the bacterial diversity and abundance were equal in all inocula.

A last result of mesh size could be a different range of soil aggregates being supplied with the inoculum. Soil aggregates must have been filtered out on size as well, with aggregates larger than the mesh size being broken down by the filtering process ¹⁵⁷. A benefit of wet sieving is that smaller soil aggregates (<2 mm) are typically more accurately represented in the sieved soil as opposed to dry sieving, with aggregates <250 μm remaining largely in tact. However, mesh sizes were in the range of 2 mm – 2 μm . Therefore, all inocula were presumably equal in the abundance soil microaggregates <2 μm and lacked larger soil aggregates(>2 mm), but the same cannot be stated for intermediate aggregate sizes as they would have been degraded depending on the mesh size that was used. The breakdown of soil aggregates in general provides the soil biota with higher levels of resources in their establishing period, which could benefit copiotrophic bacteria, taxa which excel at monopolising nutrient pools ¹⁵⁸. However, since aggregates >2 mm are broken down in all complexity levels, we expect that the additional organic matter release of smaller aggregates would have been negligible in comparison. This can be confirmed by Bach et al. (2016) who found that especially the disturbance of larger soil aggregates (>2 mm) boosted enzymatic activity and thus performance of the general microbial communities ¹⁵⁹. This elevated activity should be present at a similar rate across the complexity gradient in our experiment, indicating relatively equal conditions. This interaction is however something to keep in mind when comparing our results with natural conditions or experiments which disturbed soil aggregates to a lower degree.

Considering the dynamics described above, there is reason to suspect that the observed differences in our results are at least partly driven by a nuanced difference in methods per complexity level. However, there are still indications which show that soil biotic complexity further affected bacterial communities.

In case these differences in methods would be the only impacting variable, then we would expect that ASV richness and diversity would be the lowest in Min for all grassland sites. Yet, the general pattern for ASV richness shows an insignificant difference between Max and Min, while that of Min is still significantly decreased compared to both High and Low. This becomes even more clear when regarding specific grassland patterns, as HD and IT both lack a significant difference in alpha diversity between Max and Min, while HW actually showed an increased alpha diversity in Min compared to Max.

Considering community composition, the similarity pattern would be the following if exclusively determined by sieving precision: an insignificant difference between Max and High (as their mesh sizes should allow passage of all bacteria), which would share most similarity with Low, which would be the most similar complexity level for Min. The results of the pairwise PERMANOVA analyses strongly contrast with this theoretical pattern, providing further evidence that sieving precision did not exclusively shape bacterial communities across the complexity gradient. First, Max and High are significantly different in all grassland sites. Further, the pattern observed in three out of five grasslands, where Min showed most similarity to High, then Max and then Low is in sheer contrast with the aforementioned pattern.

It could be argued that mesh sizes selectively filtered out bacterial taxa if they are large enough and this could in theory explain for instance the reduction in relative abundance of nitrifiers and their ASV representation. However, since most nitrifiers were archaeal and these are considerably smaller than the complete set of mesh sizes ($<1\ \mu\text{m}$), they should remain unaffected¹⁶⁰. Nitrifiers should especially not be affected by the mesh sizes used for Max and High, while there still was a significant decrease in potential nitrification.

Considering the combined weight of all the dynamics described above, it can be concluded that observed differences in bacterial communities, prokaryotic functioning and plant productivity were primarily driven by soil biotic complexity.

5. CONCLUSION

The aim of this research was to study the impact of a reduced soil biotic complexity on ecosystem functioning in an otherwise intricate and highly connected soil system. We hypothesised that bacterial community composition, prokaryotic nitrogen cycling and plant productivity would be affected by a decreasing complexity gradient in such a way that the overall system destabilises.

All studied facets were affected. Bacterial communities were significantly different in soil systems within a varying range of complexity. A majority of the dominant phyla and genera saw responses in their relative abundance in the community. Most of these shifts in genera are proposed to negatively affect the ecosystem as functions associated with plant productivity would decline in efficiency. Although some of these genera responded positively to a reduced biotic complexity.

Prokaryotic nitrogen cycling became less efficient, which we suggested was in function of a degraded decomposition network. Especially potential nitrification was affected, as it almost concomitantly decreased with decreasing complexity level. Surprising was the overwhelming contribution of Archaeal nitrifiers. We briefly suggested that their performance could be linked to biotic complexity even more strongly, which makes a better understanding of their contribution a crucial pursuit in future research. Denitrification was impacted as well, likely in response to nitrification. Potential nitrogen fixation was not impacted.

We propose that functional redundancy helped stabilise potential nitrogen fixation as its ASV representation was relatively higher compared to that of the other two functions and stayed more constant with a decreasing biotic complexity. However, we suggest that individual nitrogen fixers are in fact impacted, but with more or less complementary responses, ultimately stabilising the potential nitrogen fixation.

Plant productivity saw a decline specifically in the performance of herbs, while grasses were largely unaffected. We expect that this will reduce plant species diversity in grasslands in the long run. Root

biomass increased in less complex soil systems, perhaps as a stress response or the absence of phytophagous nematodes.

Overall, different general responses were similar to multiple specific grassland responses, notably the nitrogen cycling functions and differentiation of bacterial communities in soil systems of different biotic complexity. However, different grasslands noticeably differed in their exact responses, most notably the changes in bacterial community composition which either formed a gradual gradient in accordance with soil biotic complexity or a more complex pattern in which the bacterial communities in the least complex soil communities shared relatively more similarity with those in the most complex soil communities than with intermediate soil communities. We proposed that this latter pattern could have been the effect of top-down regulation.

In conclusion, our results highlight the efficiency of a phylogenetically and functionally diverse soil system. The disruption of this system imbalances dynamics between organisms, resulting in vastly different bacterial communities, limiting functional potential of nitrogen cycling and plant productivity, likely to the detriment of the ecosystem.

6. ACKNOWLEDGEMENTS

I would like to thank the research group PLECO for supporting me during my master's thesis. I was and still am pleasantly surprised how kind and helpful everyone within the research group is. A few people in particular are of course Erik and Dajana, but also Miguel, Lorène, Matteo, Coline and Jonas.

Thank you to Erik Verbruggen, my supervisor, whom peers highly recommended as a promoter for my master's project. I can only further endorse him, as he has shown to be devoted, humble, and always ready to help, both regarding my master's project and beyond

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Especially, I will be forever grateful for Dajana. I have nothing but respect for her. Thank you for guiding me, entrusting me with responsibilities from an early stage, expecting high quality, being available almost 24/7 for my ever-burning questions and most of all, being kind. I would like to make sure you realise the impact you have had on my life and my future prospects in research.

Also thank you to my family and friends, who supported me immensely during the course of my studies in general.

7. APPENDIX

Supplement model descriptions

Significant responses which were not reported in the main results are compiled here.

Alpha diversity

ASV richness:

Grassland BZ showed a significant decrease in Low ($t_{27}=-2.142$; $p<0.042$) and Min ($t_{27}=-4.313$; $p<0.000192$) compared to Max. Grassland Ger showed a significant decrease in Min compared to Max ($t_{34}=-4.67$; $p<4.58e-05$). HW showed significant increases compared to Max in High ($t_{35}=2.514$; $p<0.017$), Low ($t_{34}=6.947$; $p<4.47e-08$) and Min ($t_{34}=3.088$; $p<0.0039$).

ASV Diversity:

Grassland BZ showed a significant decrease in Min ($t_{27}=-3.734$; $p<0.00089$) compared to Max. Grassland GER showed a significant increase in Low ($t_{34}=2.136$; $p<0.00089$) and decrease in Min ($t_{34}=-4.330$; $p<0.000125$) compared to Max. HW showed a significant increase in Low compared to Max ($t_{34}=4.09$; $p<0.0003$).

ASV representation

Nitrification

Grassland IT showed significantly fewer nitrifying ASVs in all reduced complexity levels compared to Max (High: $z=-3.028$; $p<0.0025$ | Low: $z=-3.874$; $p<0.00011$ | Min: $z=-4.652$; $p<3.28e-06$). BZ and HW only showed a significant decrease in Min (resp. $z=-2.154$; $p<0.03$ | $z=-3.190$; $p<0.0015$). HD showed a decrease both in Low ($z=-3.641$; $p<0.0003$) and Min ($z=-4.868$; $p<1.13e-06$), while GER only in Low ($z=-2.296$; $p<0.022$).

Potential functioning

Nitrification

Grassland BZ had a decreased relative abundance of nitrifiers in Low ($t_{27}=-3.281$; $p<0.003$) and in Min ($t_{27}=-4.236$; $p<0.000327$) compared to Max. Grassland GER had a decreased relative abundance of nitrifiers in High ($t_{34}=-3.061$; $p<0.0043$) and in Low ($t_{34}=-3.374$; $p<0.0019$) compared to Max. Grassland HD had a decreased relative abundance of nitrifiers in High ($t_{36}=-3.087$; $p<0.00388$), in Low ($t_{36}=-4.953$; $p<1.73e-05$) and in Min ($t_{36}=-5.866$; $p<1.05e-06$) compared to Max. Grassland HW had a decreased relative abundance of nitrifiers in High ($t_{35}=-5.365$; $p<5.31e-06$), in Low ($t_{35}=-3.136$; $p<0.0034$) and in Min ($t_{35}=-7.696$; $p<4.94e-09$) compared to Max. Grassland IT had a decreased relative abundance of nitrifiers in High ($t_{35}=-9.258$; $p<6.13e-11$), in Low ($t_{35}=-9.420$; $p<3.95e-11$) and in Min ($t_{35}=-11.941$; $p<6.73e-14$) compared to Max.

Denitrification

Grassland BZ had a decreased relative abundance of denitrifiers in Min compared to Max ($t_{27}=-2.133$; $p=0.0422$). Grassland HW had an increased relative abundance of denitrifiers in Low compared to Max ($t_{35}=2.267$; $p=0.0297$). Grassland IT had a decreased relative abundance of denitrifiers in Low ($t_{35}=-2.452$; $p=0.01933$) and in Min ($t_{35}=-3.191$; $p=0.00299$) compared to Max.

Plant Productivity

Litter

Grassland BZ had an increase in litter in Low compared to Max ($t_{36}=2.595$; $p=0.0136$). Grassland GER had a decrease in litter in Low compared to Max ($t_{36}=-2.672$; $p=0.0113$). Grassland HW had a decrease in litter in Min compared to Max ($t_{36}=-2.093$; $p=0.0435$).

Herbs

LMM for Herbs (with normality of residuals corrected with log transformation) was significantly decreased by ~ 0.2 g ($\sim 30\%$) in High ($t_{192}=-2.246365$; $p=0.0258$) and by ~ 0.4 g ($\sim 58\%$) in Min ($t_{192}=-5.401377$; $p<0.00001$). A post-hoc Tukey test showed that Min was significantly lower compared to all other complexity levels, with no further significant pairwise comparisons.

Linear models were omitted

Herb:Grass ratio (H:G)

Grassland IT had a decrease in H:G in Min compared to Max ($t_{36}=-2.226$; $p=0.0323$). Grassland GER had a decrease in H:G in Low ($t_{36}=-2.082$; $p=0.044474$) and in Min compared to Max ($t_{36}=-3.712$; $p=0.000691$).

Roots

Root biomass (LMM) is significantly increased by 0.38 g ($\sim 10\%$) both in Low ($t_{192}=2.045640$; $p=0.0422$) and Min ($t_{192}=2.031487$; $p=0.0436$). Post-Hoc Tukey test did not find any significant differences.

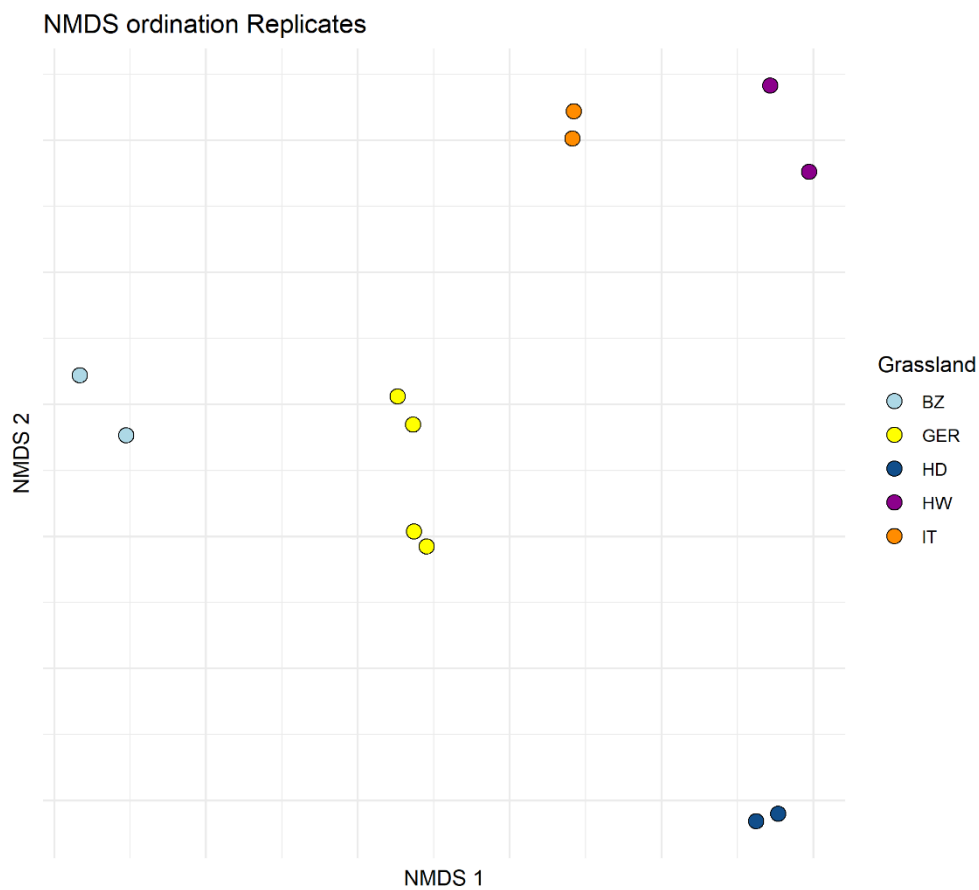
Linear models were omitted

Root:shoot ratio

Grassland GER had an increase in root:shoot in Low ($t_{36}=4.027$; $p=0.0000278$) and in Min compared to Max ($t_{36}=-3.302$; $p=0.002173$). Grassland HD had an increase in root:shoot in Low ($t_{36}=3.252$; $p=0.00249$) and in Min compared to Max ($t_{36}=4.968$; $p=1.66e-05$).

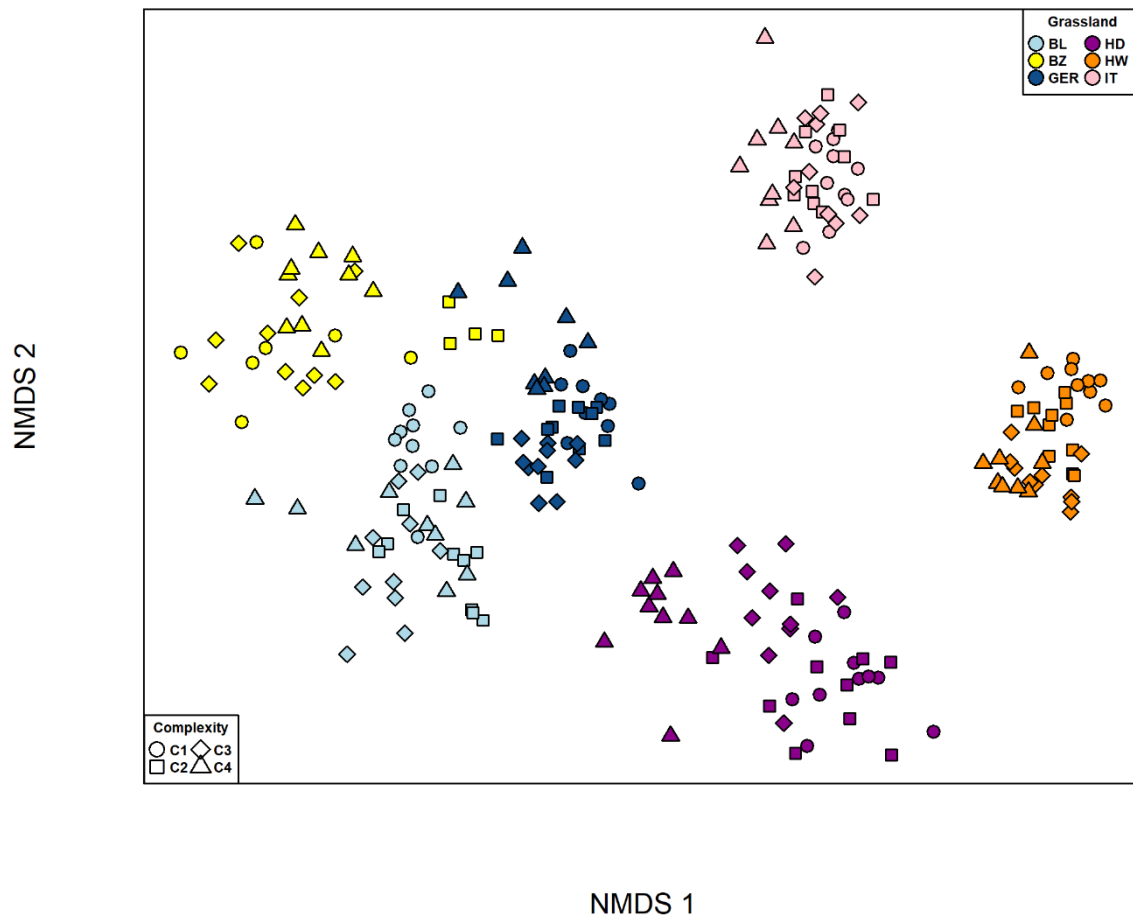
Appendix 1 **Samples per grassland per complexity level** . This table gives an overview of the number of samples which were considered to be successful and were used in further analysis. Original sample size was 10 for each grassland-complexity level combination.

		Grassland site				
		BZ	GER	HD	HW	IT
Complexity Level	Min	10	9	10	9	9
	Low	10	10	10	10	10
	High	4	10	10	10	10
	Max	7	9	10	10	10

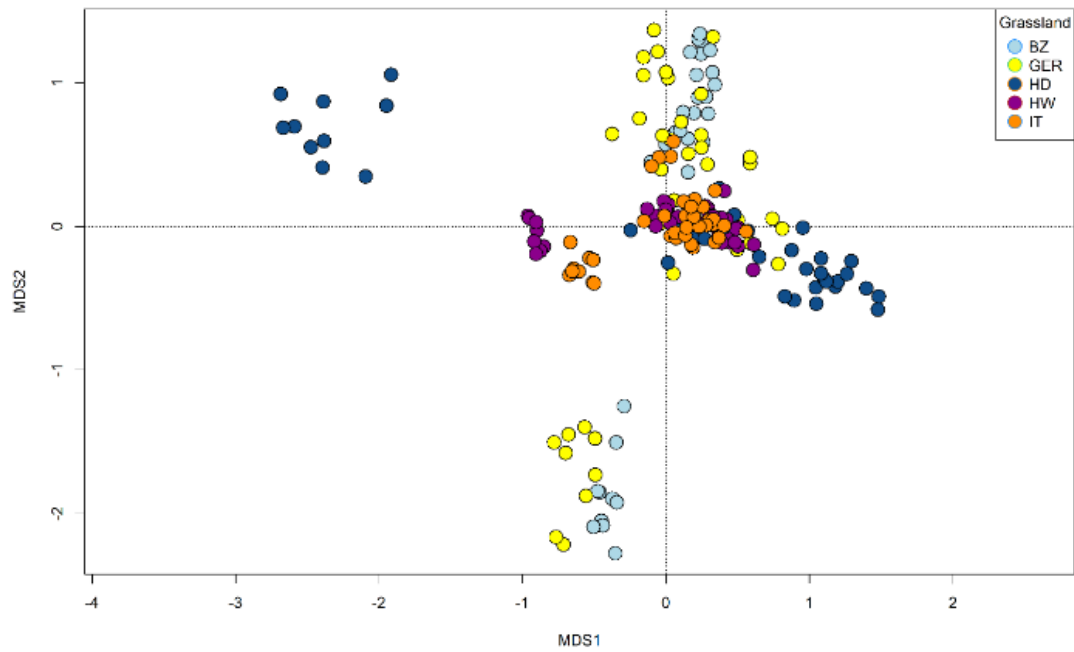


Appendix 2. NMDS ordination replicates. This ordination visually confirms the repeatability and relative success of the DNA amplification. Stress<0.2.

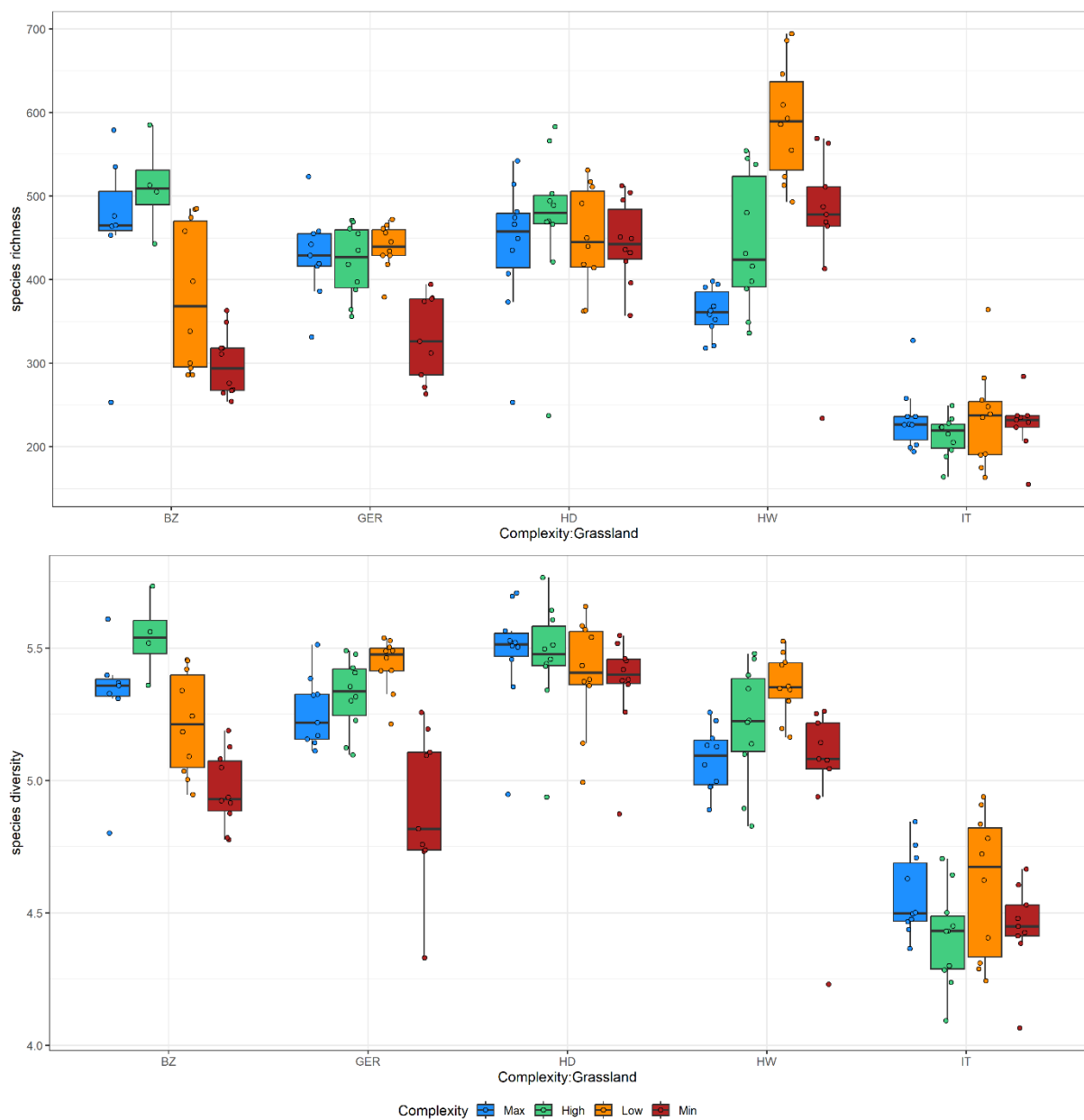
NMDS ordination Grassland:Complexity



Appendix 3. NMDS ordination dataset. This NMDS ordination combines the samples of all grasslands. The ordination was not constrained to any variable. It shows the strong clustering per grassland, which is why dbRDA was used to more closely compare the complexity levels. Note that in this figure a sixth grassland is included “BL” which is not part of the data in this thesis and that grassland colouring does not follow the colour code of this thesis. Complexity level codes conform to the following: C1=Max, C2= High, C3=Low and C4=Min.



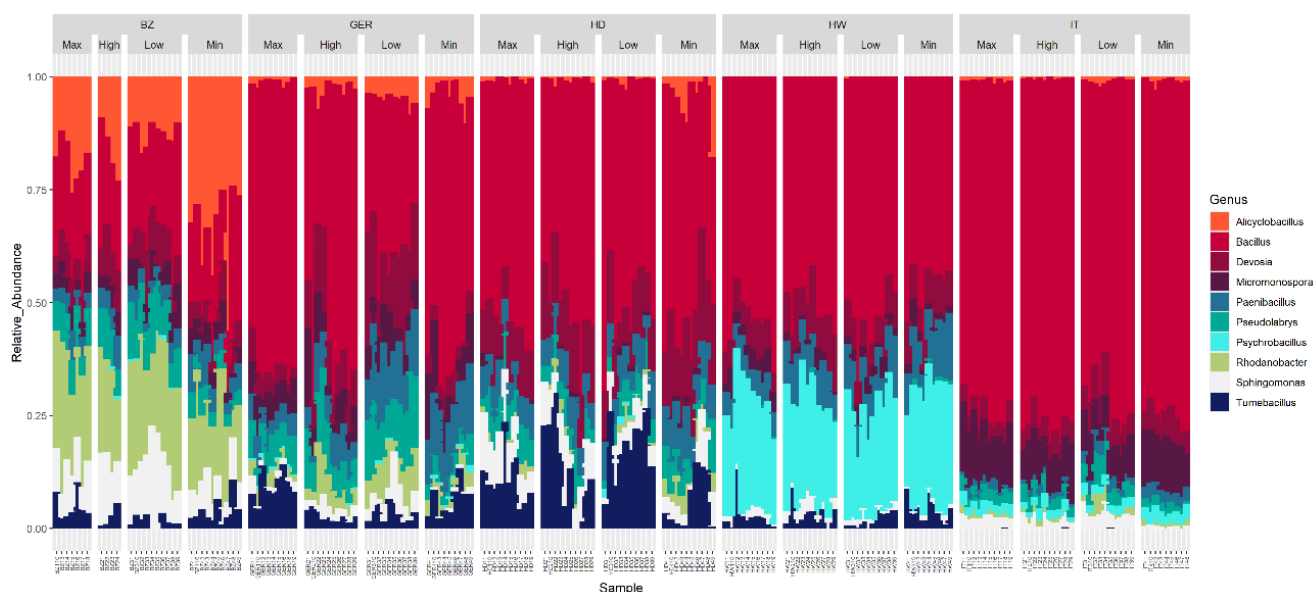
Appendix 4. dbRDA ordination grassland indication. This ordination is the exact same as presented in Figure 3, but shows colouring based on grassland. The ordination visualises how not all grassland variation is corrected for and/or that bacterial communities of different grasslands seem to have differing responses to soil communities of different soil biotic complexity.



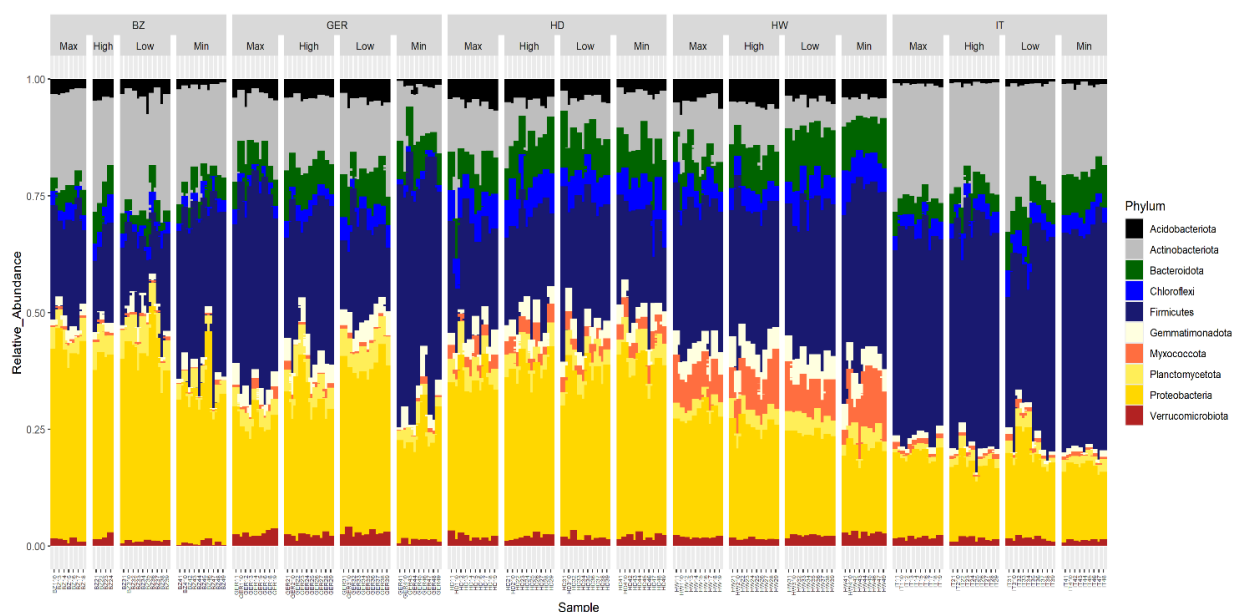
Appendix 5. Alpha Diversity across the Complexity levels per Grassland. This figure shows the variation in ASV richness (Top) and ASV diversity based on Shannon-Index (Bottom) for every Complexity level per Grassland.

Appendix 6. Results pairwise PERMANOVA analyses. This table summarises the results of the pairwise PERMANOVA analyses per grassland, comparing complexity levels within the grassland with each other. The results show that complexity levels contain significantly different bacterial communities. A different model was run per grassland.

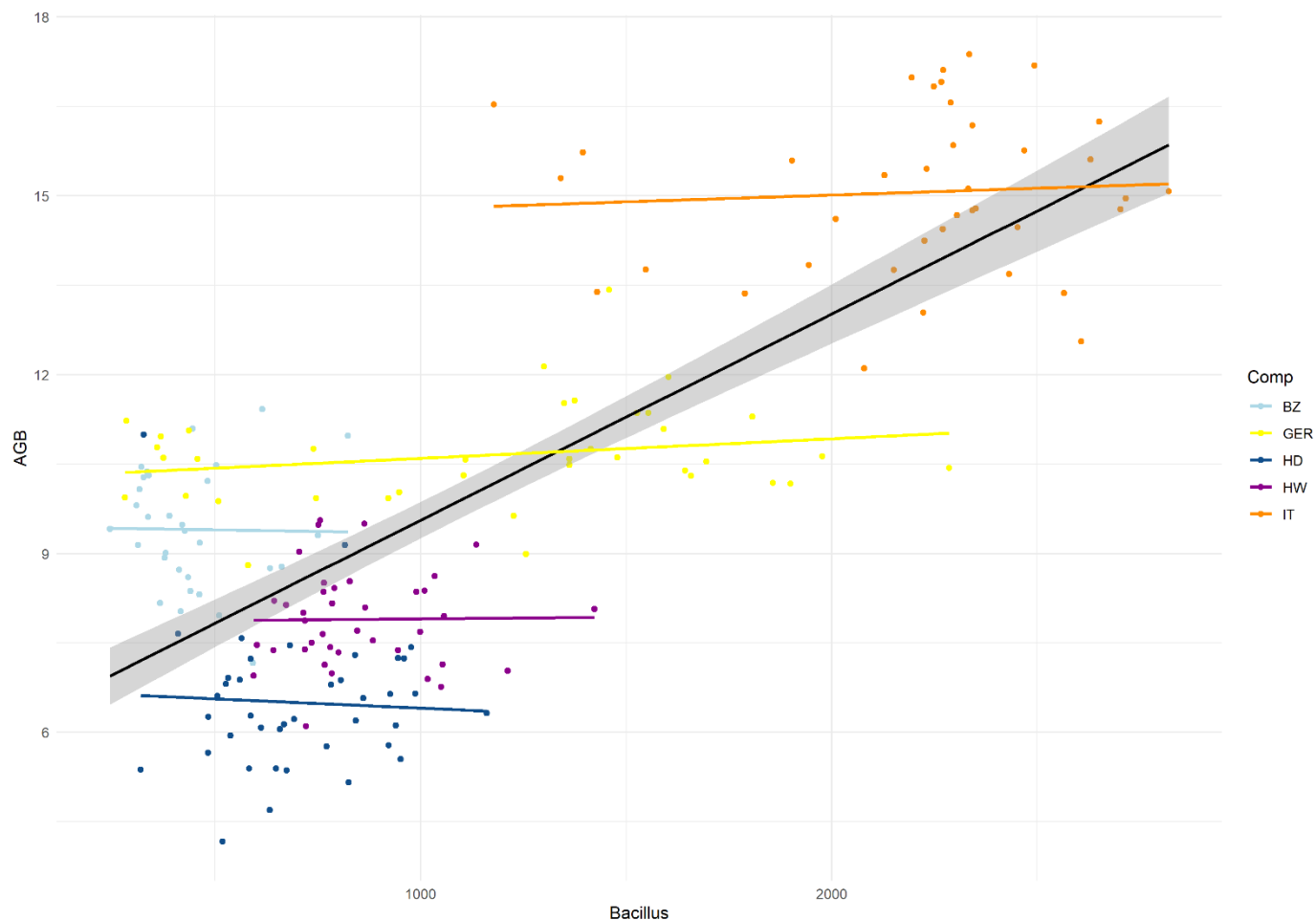
<i>Grassland</i>	<i>pairs</i>	<i>Df</i>	<i>R²</i>	<i>Adjusted p</i>
IT	Max vs High	1	0.2167097	6,00E-04
	Max vs Low	1	0.2601547	6,00E-04
	Max vs Min	1	0.3546931	6,00E-04
	High vs Low	1	0.1517871	0.015
	High vs Min	1	0.3093164	6,00E-04
	Low vs Min	1	0.2973098	6,00E-04
HW	Max vs High	1	0.213244	6,00E-04
	Max vs Low	1	0.260751	6,00E-04
	Max vs Min	1	0.3836048	6,00E-04
	High vs Low	1	0.1980597	6,00E-04
	High vs Min	1	0.3742216	6,00E-04
	Low vs Min	1	0.2872941	6,00E-04
HD	Max vs High	1	0.08159701	0.0066
	Max vs Low	1	0.16621649	6,00E-04
	Max vs Min	1	0.34495052	6,00E-04
	High vs Low	1	0.11496286	6,00E-04
	High vs Min	1	0.31377005	6,00E-04
	Low vs Min	1	0.27450752	6,00E-04
GER	Max vs High	1	0.234768	6,00E-04
	Max vs Low	1	0.3944411	6,00E-04
	Max vs Min	1	0.3232694	6,00E-04
	High vs Low	1	0.1350585	0.0054
	High vs Min	1	0.2879767	6,00E-04
	Low vs Min	1	0.3536504	6,00E-04
BZ	Max vs High	1	0.2291781	0.0198
	Max vs Low	1	0.1782082	6,00E-04
	Max vs Min	1	0.3614146	6,00E-04
	High vs Low	1	0.1515444	0.0138
	High vs Min	1	0.3165736	0.0066
	Low vs Min	1	0.3021522	6,00E-04



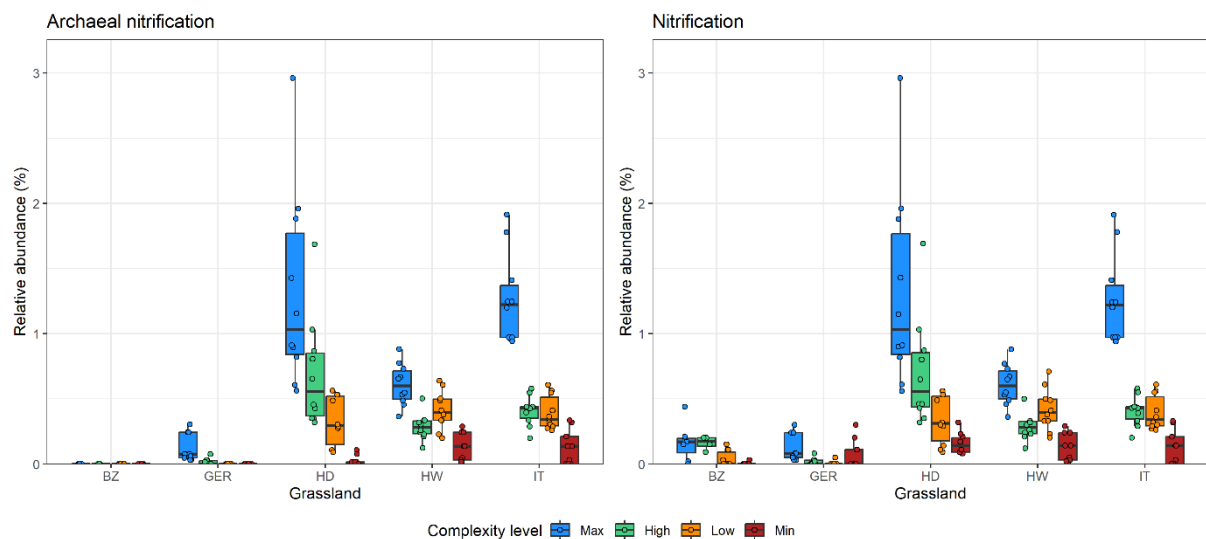
Appendix 7. Representation of the 10 dominant genera per sample. This plot shows the proportions in which the dominant genera are represented in each sample. Only the 10 dominant genera are taken into account and thus the y axis shows the abundance relative to the aggregation of dominant genera, not the entire bacterial community of the sample. Samples are grouped per complexity level and then per grassland. The X axis represents all samples



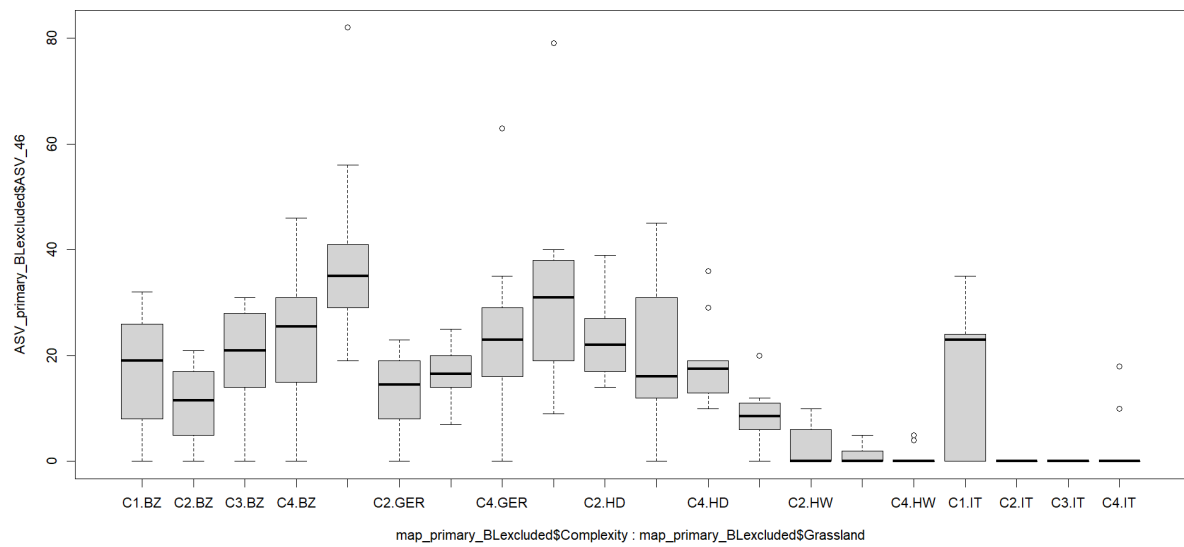
Appendix 8. Representation of the 10 dominant phyla per sample. This plot shows the proportions in which the dominant phyla are represented in each sample. Only the 10 dominant phyla are taken into account and thus the y axis shows the abundance relative to the aggregation of dominant phyla, not the entire bacterial community of the sample. Samples are grouped per complexity level and then per grassland. The X axis represents all samples



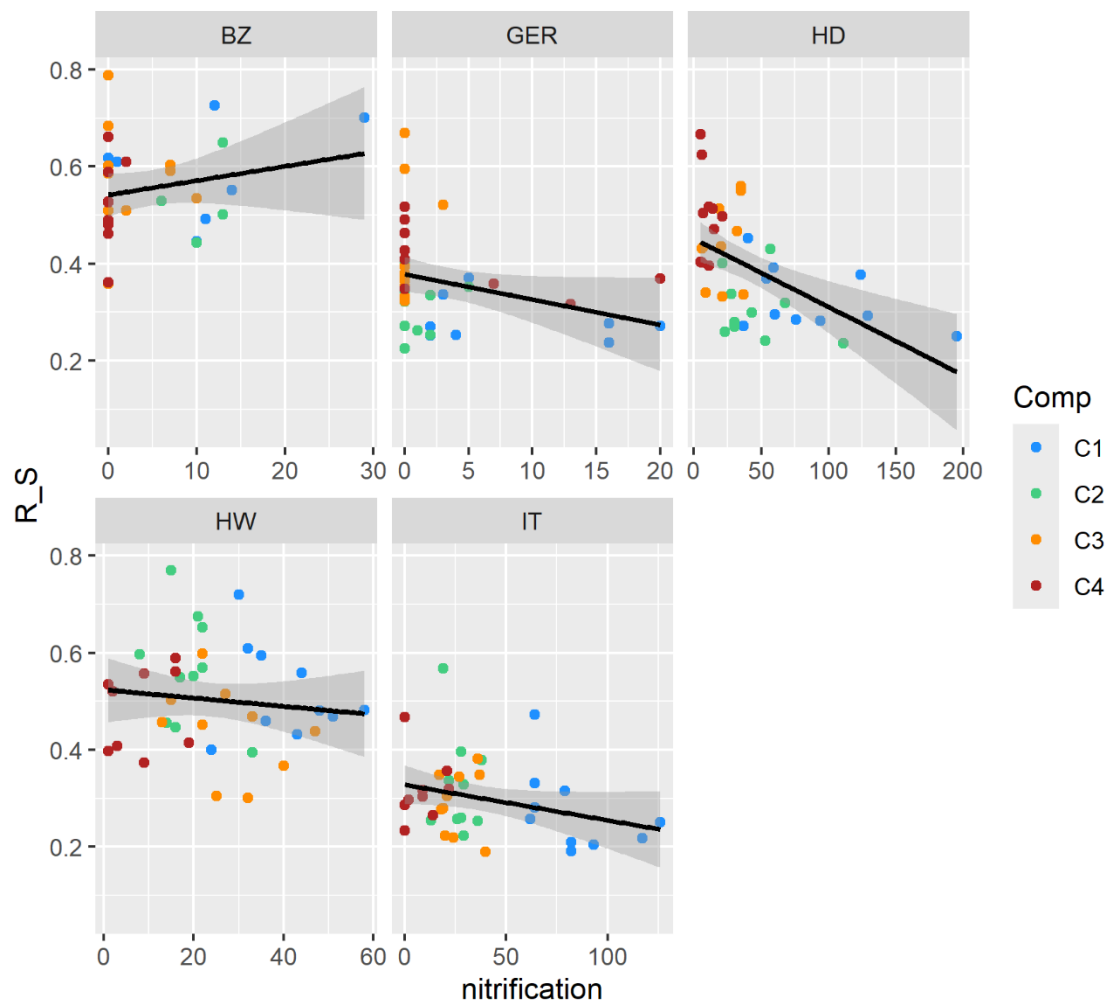
Appendix 9. AGB in function of *Bacillus* reads. This plot highlights the argument in section 4.1 (subheading *dominant genera*) in the discussion which suspects that the relative abundance of *Bacillus* in the soil strongly contributes to soil fertility and indirectly to plant productivity. Note that the overall relation between Aboveground biomass (AGB) is highly correlated in general, but not within a grassland (complexity level not taken into account). X axis is expressed as total number of rarefied reads. Y axis shows the total amount of AGB expressed in gram.



Appendix 10. Archaeal and total nitrification. These boxplots visualise the potential nitrification based on Archaeal ASVs (**Left**) and total potential nitrification (**Right**) as presented previously in the main results. Archaeal nitrification outweighs bacterial nitrification by far (93% of nitrifying reads). Archaeal nitrification seems to decline in a more pronounced pattern than total nitrification. Note that bacterial nitrification increases in complexity level Min for HD and GER. BZ is the only grassland which shows exclusively bacterial nitrification.



Appendix 11. Relative abundance of *Bradyrhizobium elkanii*. These boxplots show the relative abundance (represented as total rarefied reads) of *Bradyrhizobium elkanii*, ASV 46 in our dataset, across the complexity gradient in different grasslands. This species is a potential nitrogen fixer, a root nodulating symbiont, which is most notably abundant in complexity level Max (here presented as “C1”). This pattern further supports the hypothesis that different nitrogen fixating ASVs thrive in different levels of the complexity gradient. See 4.2 Nitrogen cycle (Subheading *Nitrogen fixation*).



Appendix 12. Root-shoot ratio in function of nitrification potential per grassland. These scatter plots show the root-shoot ratio in function of total nitrifier reads. Note that most grasslands show a negative correlation between the two variables, although strongest in HD. Comp=Complexity level (C1=Max, C2=High, C3=Low, C4=Min). note that x scales differ and should not be compared with each other.

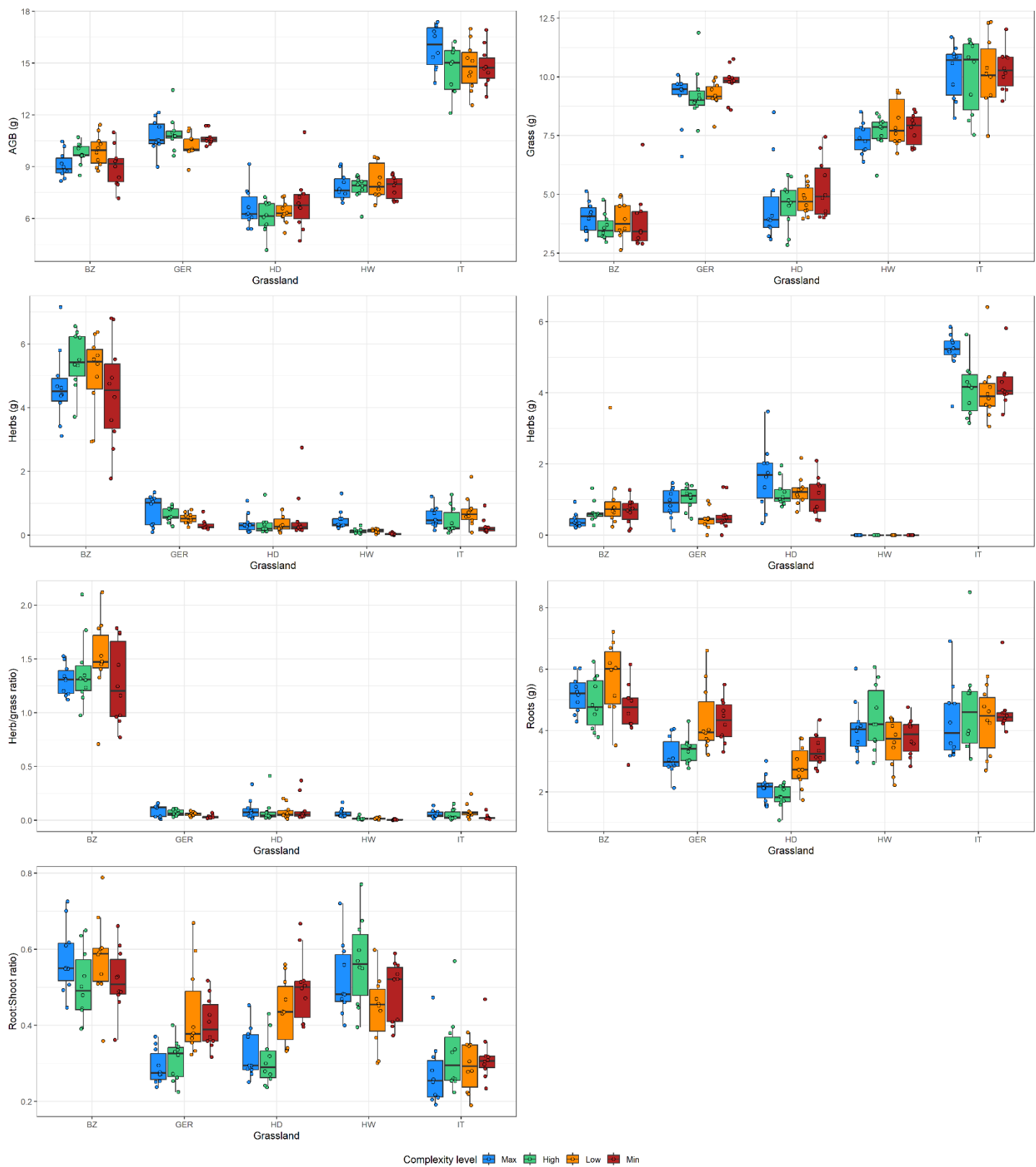
Appendix 13. Plant species per grassland. This table summarises the 10 different species which were selected per grassland based on their native status and relative dominance in the site. Group indicates whether the species was a grass or a herb. For further clarification, see section 2.1 Experimental setup (subheading *Plant community selection*)

<i>Grassland</i>	<i>Species</i>	<i>Group</i>	<i>Grassland</i>	<i>Species</i>	<i>Group</i>
IT	<i>Trifolium pratense</i>	Herb	HD	<i>Festuca vaginata</i>	Grass
	<i>Cynosurus cristatus</i>	Grass		<i>Stipa borysthena</i>	Grass
	<i>Arrhenatherum elatius</i>	Grass		<i>Melica transsylvanica</i>	Grass
	<i>Phleum pratense</i>	Grass		<i>Cynodon dactylon</i>	Grass
	<i>Dactylis glomerata</i>	Grass		<i>Bromus tectorum</i>	Grass
	<i>Trifolium repens</i>	Herb		<i>Crepis rheoadifolia</i>	Herb
	<i>Rumex acetosa</i>	Herb		<i>Linum austriacum</i>	Herb
	<i>Taraxacum officinale</i>	Herb		<i>Silene otites</i>	Herb
	<i>Chaerophyllum aureum</i>	Herb		<i>Vicia angustifolia (sativa)</i>	Herb
GER	<i>Poa pratensis</i>	Grass	BZ	<i>Chondrilla juncea</i>	Herb
	<i>Holcus lanatus</i>	Grass		<i>Agrostis capillaris</i>	Grass
	<i>Agrostis capillaris</i>	Grass		<i>Anthoxanthum odoratum</i>	Grass
	<i>Festuca rubra</i>	Grass		<i>Molinia caerulea</i>	Grass
	<i>Alopecurus pratensis</i>	Grass		<i>Lotus pedunculatus</i>	Herb
	<i>Plantago lanceolata</i>	Herb		<i>Potentilla erecta</i>	Herb
	<i>Rumex acetosa</i>	Herb		<i>Silene flos-cuculi</i>	Herb
	<i>Poa pratensis</i>	Grass		<i>Trifolium repens</i>	Herb
	<i>Glechoma hederacea</i>	Herb		<i>Succisa pratensis</i>	Herb
HW	<i>Stellaria graminea</i>	Herb		<i>Holcus lanatus</i>	Grass
	<i>Vicia angustifolia (sativa)</i>	Herb		<i>Festuca rubra</i>	Grass
	<i>Deschampsia caespitosa</i>	Grass			
	<i>Dactylis glomerata</i>	Grass			
	<i>Briza media</i>	Grass			
	<i>Arrhenatherum elatius</i>	Grass			
	<i>Alopecurus pratensis</i>	Grass			
	<i>Lysimachia vulgaris</i>	Herb			
	<i>Galium verum</i>	Herb			
	<i>Betonica officinalis (Stachys off.)</i>	Herb			
	<i>Dorycnium germanicum</i>	Herb			
	<i>Ononis spinosa</i>	Herb			

Appendix 14. Plant species number per grassland. This table gives an overview of the mean number of grass and herb species that managed to establish in microcosms of different grasslands and complexity levels. Species were counted based on if any biomass was identified as the species, not taking into account the amount. Standard deviation is given. This data is part of the encompassing project which gathered biomass per plant species as much as possible. The data is not yet analysed at large extent.

To illustrate the hypothesis in 4.3 Plant Productivity (subheading: *aboveground biomass*) of the discussion, note how herb species in HW decline with complexity level, while grass species remain constant.

<i>Grassland</i>	<i>Type</i>	<i>Max</i>	<i>High</i>	<i>Low</i>	<i>Min</i>
BZ	Grass	3.8 ± 0.42	3.8 ± 0.42	3.8 ± 0.42	3.7 ± 0.48
BZ	Herbs	3.2 ± 0.79	3 ± 0.47	3.1 ± 0.88	2.6 ± 0.97
GER	Grass	3.6 ± 0.84	3.4 ± 0.5	3.7 ± 0.82	3.4 ± 0.52
GER	Herbs	3 ± 0.82	4.1 ± 0.74	3.6 ± 0.84	3.4 ± 0.97
HD	Grass	3.8 ± 0.42	3.7 ± 0.48	3.9 ± 0.32	4 ± 0
HD	Herbs	2.5 ± 0.71	2.8 ± 0.42	3 ± 0	2.5 ± 0.53
HW	Grass	3 ± 0	3 ± 0	3 ± 0	3 ± 0
HW	Herbs	4.1 ± 0.57	3.5 ± 0.53	3.6 ± 0.97	2.2 ± 0.92
IT	Grass	2.9 ± 0.32	2.9 ± 0.32	3 ± 0	3 ± 0
IT	Herbs	1.7 ± 0.48	1.7 ± 0.48	1.8 ± 0.42	1.4 ± 0.69



Appendix 15. Plant productivity parameters per grassland per complexity level. This multipaneled figure shows the boxplots for all considered plant productivity parameters per grassland per complexity level. Y axes are either represented as ratios or in grams.

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