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Human impact on bacterial communities of agricultural soils and its implications for sustainable use

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Summary

Human influences on the environment have risen to such intensity that they justify the transition to a new geological epoch, the Anthropocene. The starting time of this new epoch is still debated and there are suggestions between the Neolithic period and the mid-twentieth century. However, human influence at the landscape level is undoubted. In Europe in particular, humans have been imprinting natural landscapes with cultural landscapes for centuries. Soils used for agriculture are particularly exposed to anthropogenic factors. Common soil cultivation techniques can have a major influence on the bacterial community in the soil. The aim of this doctoral thesis is therefore to qualitatively and quantitatively assess the influence of land-use changes on the composition of the soil bacterial community. Based on potential key species within the bacterial community, an assessment of the ecological and economic sustainability of these land use changes is made. In order to gain these insights, ideal sites are locations that underwent several changes in their form of use within a few years and for which several ages were available in the form of a chronosequence. Thus, former opencast mining sites, from the status of active site restoration to multi-decadal agricultural use, are an object of investigation for this doctoral thesis. Here it is shown that each phase of restoration with its own management variant has its own bacterial community with typical representatives. The extent of these changes is comparable to those during seasonal fluctuations. Particularly noteworthy, however, is the observation that key taxa of the soil bacterial community and plant growth-promoting organisms also show preferences for the different phases. This phase preference is exemplified by the groups of Rhizobiales and *Streptomyces*; both ecologically (and potentially also economically) important bacterial groups that can apparently be influenced by changes in use. The effects of these changes are not yet known, especially on the partly highly specialised interactions with our crops. However, more in-depth knowledge of these interactions would be desirable to ensure sustainable use.

As soon as the effects of an agricultural measure on the soil bacteria are known, it could also be used to change the properties of the bacterial community in a targeted manner. In the second part of this work, this effect is explored using the example of soil improvement by means of organic substances. This analysis is carried out on the areas of a long-term fertilisation trial, where the effects of farmyard manure as an animal substance and crop residues as a plant material are compared with each other. While the long-term integration of farmyard manure into the management system produced a change in the bacterial community and positively influenced bacterial diversity, the use of crop residues did not produce such a change. In particular, three families of the order Bacillales, namely Planococcaceae, Thermoactinomycetaceae and Turicibacteraceae, benefited from manure. Plant growth-promoting bacteria are known from each of these families. However, manure and crop residues were equally likely to maintain the

complexity of bacterial co-occurrence networks at a higher level compared to control conditions. This mitigating effect could play a crucial role in the sustainability of farming, as it is associated with more robust ecosystems and thus more stable yields.

Taken together, the results of this doctoral thesis offer insights into how human interventions in the context of e.g. restoration measures and agriculture affect bacterial soil communities and provide indications of what consequences might be expected. In addition, there are links for future research projects that address the existing gaps in knowledge, especially in the area of microbial interactions at the organismic level, but also the effects of other management variants.

Zusammenfassung

Menschliche Einflüsse auf die Umwelt haben eine solche Intensität erreicht, dass sie den Übergang in eine neue geologische Epoche, das Anthropozän, rechtfertigen. Über den Startzeitpunkt dieser neuen Epoche wird weiterhin diskutiert und es gibt Vorschläge zwischen dem Neolithikum und der Mitte des zwanzigsten Jahrhunderts. Unzweifelhaft ist hingegen der menschliche Einfluss auf Landschaftsebene. Insbesondere in Europa hat der Mensch seit Jahrhunderten natürliche Landschaften durch Kulturlandschaften überprägt. Landwirtschaftlich genutzte Böden sind anthropogenen Einflussfaktoren besonders ausgesetzt. Gebräuchliche Techniken der Bodenbearbeitung können dabei großen Einfluss auf die Bakteriengemeinschaft im Boden ausüben. Ziel dieser Dissertation ist es daher, den Einfluss von Landnutzungsänderungen auf die Zusammensetzung der bakteriellen Bodengemeinschaft qualitativ und quantitativ zu bewerten. Anhand von potenziellen Schlüsselarten innerhalb der Bakteriengemeinschaft soll eine Bewertung der ökologischen und ökonomischen Nachhaltigkeit dieser Landnutzungsänderungen vorgenommen werden. Um diese Einblicke zu gewinnen waren Standorte ideal, die innerhalb weniger Jahre mehrfach eine Änderung ihrer Nutzungsform erfuhren und für die mehrere Altersstufen in Form einer Chronosequenz vorlagen. So sind ehemalige Tagebauflächen, vom Status der aktiven Flächenrenaturierung bis hin zu einer mehrdekadigen landwirtschaftlichen Nutzung, ein Untersuchungsgegenstand dieser Doktorarbeit. Hierbei zeigte sich, dass jede Phase der Renaturierung mit der ihr eigenen Managementvariante eine eigene bakterielle Gemeinschaft mit typischen Vertretern besitzt. Der Umfang dieser Veränderungen ist vergleichbar mit denen während jahreszeitlicher Schwankungen. Besonders hervorzuheben ist aber die Beobachtung, dass auch Schlüsseltaxa der bakteriellen Bodengemeinschaft und pflanzenwachstumsfördernde Organismen Präferenzen für die verschiedenen Phasen aufweisen. Exemplarisch konnte dies an den Gruppen der Rhizobiales und *Streptomyces* gezeigt werden; beides ökologisch (und potenziell auch ökonomisch) wichtige Bakteriengruppen, die offenbar durch Nutzungsveränderungen beeinflusst werden. Die Auswirkungen dieser Veränderungen sind bislang nicht bekannt, insbesondere auf die teils hochspezialisierten Interaktionen mit unseren Nutzpflanzen. Vertiefte Kenntnisse über diese Zusammenhänge wären jedoch zu Sicherstellung einer nachhaltigen Nutzung wünschenswert.

Sobald man die Auswirkungen einer landwirtschaftlichen Maßnahme auf die Bodenbakterien kennt, könnte man diese auch dazu einsetzen, Eigenschaften der Bakteriengemeinschaft gezielt zu verändern. Am Beispiel von Bodenverbesserung mittels organischer Substanzen sollte dies im zweiten Teil dieser Arbeit erforscht werden. Dieser wurde auf den Flächen eines Langzeitdüngeversuchs durchgeführt, wo die Effekte von Stallmist als tierische Substanz und von Ernterückständen als pflanzliches Material miteinander verglichen wurden. Während die

langfristige Integration von Stallmist in die Bewirtschaftungsform eine Veränderung der Bakteriengemeinschaft hervorrief und die bakterielle Diversität positiv beeinflusste, brachte die Nutzung von Ernterückständen keine solche Veränderung hervor. Insbesondere drei Familien der Ordnung Bacillales, nämlich Planococcaceae, Thermoactinomycetaceae und Turicibacteraceae, profitierten vom Stallmist. Aus diesen Familien sind jeweils pflanzenwachstumsfördernde Bakterien bekannt. Stallmist und Ernterückstände waren jedoch gleichermaßen dazu geeignet die Komplexität bakterieller Kookkurrenznetzwerke gegenüber Kontrollbedingungen auf einem höheren Niveau zu halten. Dies könnte für die Nachhaltigkeit der Bewirtschaftung eine entscheidende Rolle spielen, da dies mit robusteren Ökosystemen und damit stabileren Erträgen assoziiert wird.

Zusammengenommen bieten die Ergebnisse dieser Doktorarbeit Erkenntnisse darüber, wie menschliche Eingriffe im Rahmen von z.B. Rekultivierungsmaßnahmen und Landwirtschaft sich auf die bakteriellen Bodengemeinschaften auswirken und geben Hinweise darauf, mit welchen Konsequenzen zu rechnen sein könnte. Zudem ergeben sich Anknüpfungspunkte für zukünftige Forschungsprojekte, die die vorhandenen Wissenslücken besonders im Bereich der mikrobiellen Interaktionen auf organismischer Ebene, aber auch der Effekte anderer Managementvarianten behandeln.

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1 Introduction

1.1 General introduction

Most, if not all, ecosystems on Earth are influenced by human actions (Foley et al. 2005; Liebhold et al. 2017; Newbold et al. 2018). This is true for the oceans and the land, for cultivated and natural landscapes and even for extreme environments such as the deep sea (Halpern et al. 2008; Egger et al. 2020). The consequences of human influence are so substantial worldwide that the transition to a new geological epoch, the Anthropocene, is being considered (Zalasiewicz et al. 2008; Waters et al. 2016). This new epoch is intended to take account of human influences on biological, atmospheric and geological processes on our planet. However, the starting time of this new epoch is subject to scientific debate (Certini & Scalenghe 2015; Monastersky 2015; Subramanian 2019). In every ecosystem, human impact is of different nature and intensity, affecting them at different scales from the regional and landscape level down to the microscopic level. In Europe, large portions of the landscape have been in use in one way or another for centuries. This usage resulted in ecosystems with long land-use histories (Poschlod 2015). Different types of land use lead to typical flora and fauna in these anthropogenic ecosystems and thus fundamentally shaped the appearance of the European scenery. The long history of this use facilitated new plant and animal communities that are now well adapted to these habitats. However, modernisation of agriculture and forestry led to the abandonment of traditional land-use practices and produces the new dilemma of these anthropogenic communities becoming endangered by losses of species diversity (Niedrist et al. 2008).

While such changes in landscape and vegetation structure that are due to land-use change, are both relatively easy to identify and to grasp, changes to the microscopic world of microorganisms were for a long time as obscure as they were hard to assess. It has long been assumed that our soils are the ecosystems with the highest biodiversity on our planet (Curtis et al. 2002). While plant roots represent a large part of the underground biomass and strongly influence the physical and chemical properties through their exudates (Hellequin et al. 2021), biodiversity in soil is predominantly determined by microorganisms. In a sample of just one gram of soil, billions of bacterial cells, tens of thousands of protists and invertebrates, and hundreds of metres of fungal hyphae can be found (Decaëns 2010; Orgiazzi et al. 2016; Geisen et al. 2018; Gupta 2020). While many thousands of soil organisms have already been identified, it is estimated that millions more species of bacteria, archaea, fungi, protists, nematodes, springtails, mites, Enchytraeidae, earthworms and macro-arthropods await discovery (Geisen et al. 2019a). This almost unimaginable diversity characterises our soils, making them an extremely complex ecosystem that continues to present challenges for its study (Fierer 2017; Rodrigo-Comino et al. 2020). For example, discussions on appropriate biological indicators of soil health have been ongoing since at

least the 1990s (Pankhurst et al. 1997); a topic that is more relevant than ever today in the context of climate change, harmful human impacts in agriculture and necessary debates on sustainability (Schloter et al. 2018; Tahat et al. 2020; Gorain & Paul 2021). The issue of soil biodiversity has also entered the political arena, where its importance for human society should be emphasised to policymakers (FAO et al. 2020).

Despite the knowledge of the special position of soils in terms of microbial diversity and activity, and despite the inherent value of healthy soils for humans (Fierer et al. 2021), it has long been difficult to study microbial processes in detail. One reason for this was that the vast majority of prokaryotic species could not yet be obtained in pure culture to describe them (Schleifer 2004). Moreover, studies based on the production of pure cultures are far too inefficient for a diversity hotspot such as soils to be able to evaluate changes in biodiversity and their causes in a meaningful way. Within the past two decades, however, alterations to the soil microbial community following human intervention could finally be assessed in more detail by technological advancements of culture-independent tooling such as fingerprinting methods or next generation sequencing. The first findings using these technologies revealed the unsurprising fact that different types of land use impacted on the microbial soil communities in a similar way to what had been known from macro-ecological investigations (Steenwerth et al. 2002). The use of these technologies allowed microbial ecologists to determine the actual extent of these microbial community changes. Microbial communities have been found to respond not only to the different land-use regimes, but also to the different land management techniques used over the course of a season (Bevivino et al. 2014). When land-use changes, e.g. due to restoration measures or by changing to a different management type, this will in turn change the composition of soil microbial communities, similar to the changes in animal and plant species abundances in macro-ecology (Allison et al. 2005).

Furthermore, culture-independent methods allowed to assess the rate at which land management affects microbial communities. Alterations of the microbial community structure and its functional properties happen gradually and may take a long time. Interestingly, the composition of the microbial community and its mineralisation rates have been shown to depend not only on the current type of land use. In fact, land-use history exerts an even greater influence (Fraterrigo et al. 2006). Especially in bacterial and archaeal communities, this effect can persist for at least several years after management changed. During this time, the respective communities change but still show differences corresponding to the previous land use (Hirsch et al. 2017). Particularly in the context of restoration, it is therefore important to consider the history of a site's land use. Even the influence of fundamental factors such as soil chemical properties or current vegetation can be modulated by land-use history (Jangid et al. 2011). This includes crop species grown on agricultural land in previous years (Babin et al. 2018). Long-term cultivation of restored land cannot

completely eliminate this effect, and the consequences of land-use change may still be visible after many years under the new land use (Yao et al. 2006). These findings give way to an even wider area of soil microbiology research that is important to tackle in order to provide environmentally sound agricultural land management solutions as well as restoration measures for the future.

Another aspect that needs to be considered, when assessing the impact of anthropogenic activities on soil microorganisms, is the role of biodiversity in the functioning of the soil ecosystem. At this point, it is worth drawing parallels with macroecology to illustrate the fundamental importance of diversity for the robustness of ecosystems. In plant communities, species diversity has been identified as an important prerequisite for ecosystem stability in the face of sudden environmental changes (MacDougall et al. 2013). Depending on the species group this effect can be mediated by the functional diversity within the community (Flynn et al. 2009). Rare species in particular contribute significantly to the range of functions that can be fulfilled by the ecosystem (Mouillot et al. 2013). In addition, high diversity can significantly reduce sensitivity to invasive species (Zavaleta & Hulvey 2004). Besides semi-natural sites, this role of biodiversity applies equally to agricultural systems, both in grassland and arable land (Dardonville et al. 2020). Most modern microbiological-ecological studies assess the diversity of the soil communities studied. Similar to species diversity enhancing adaptability in plant communities, diversity of soil microbial communities can be thought to enhance the ecological stability of soil ecosystems towards external stresses. This line of thought is suggested by research on the resistance and resilience of microbial parameters during restoration of habitats. Disturbance of a microbial community can cause it to be more sensitive to subsequent stress (Kuan et al. 2006). Depending on the nature and similarity of the applied stresses, the sensitivity of a community after the initial perturbation may be considerably higher than before (Philippot et al. 2008). Since the composition of the microbial communities usually changes in response to perturbations, ecosystem process rates may be directly affected and in turn impact on ecosystem functions (Allison & Martiny 2008). From the various microbial parameters usually measured in soil, like e.g. α-diversity, respiration or mineralisation rates, not all will respond in the same manner, since they represent different aspects of the soil microbial community. Furthermore, depending on the disturbance history, the types of disturbance applied can have different effects on the various parameters (Tobor-Kapłon et al. 2009). The microbial diversity of soil was shown to be directly linked to soil respiration rates (Bell et al. 2005; Maron et al. 2018). As such, species diversity is an important prerequisite for ecosystem functioning. Soil microbial communities with higher biodiversity are commonly assumed to have higher resistance and resilience (Bardgett & Caruso 2020). For example, soils from cropland with lower diversity have been shown to be both less resistant and less resilient to perturbations than ecosystems with a higher microbial diversity such as grasslands and natural forests (Chaer et al. 2009; Riah-Anglet et al. 2015). In essence, when a soil ecosystem is disturbed, its diversity is a

key feature that enhances its community stability as well as its functional resilience and thus promotes its sustainability in the long term (Girvan et al. 2005). However, biodiversity can have both positive and negative effects on individual components of ecosystem stability and should therefore be considered in context (Pennekamp et al. 2018).

1.2 The soil microbial community is shaped by agricultural practices

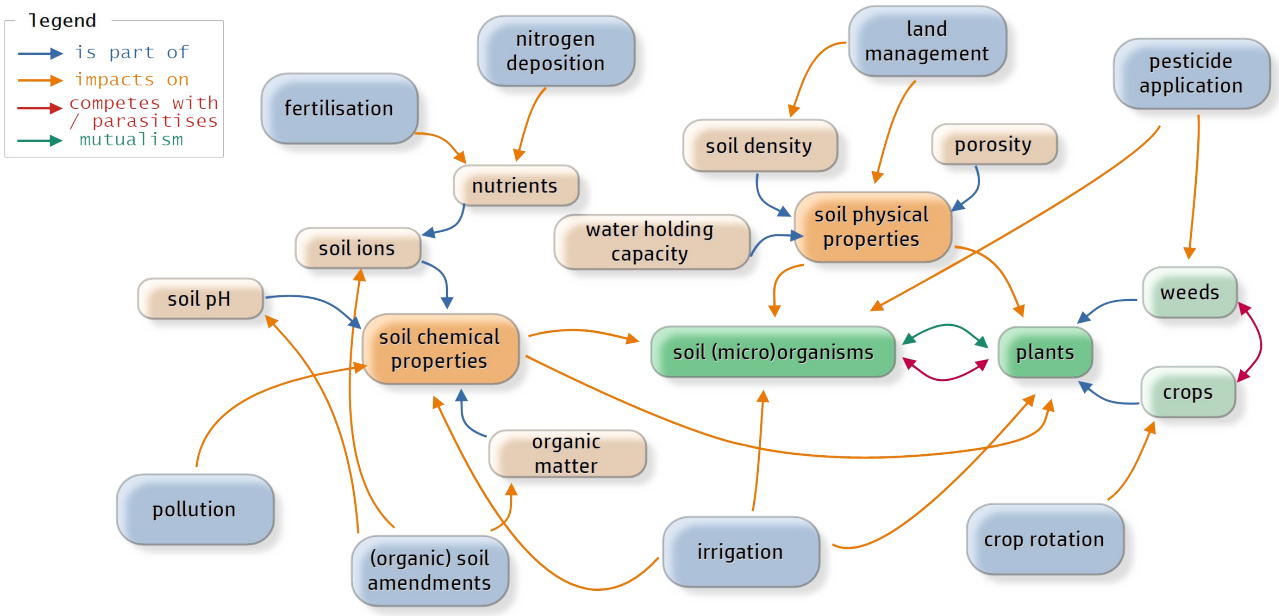

Figure 1: *Mind map of an agricultural soil system.*

The organisms within an agricultural system (green) influence each other both negatively and positively. At the same time, they themselves are influenced by various abiotic factors (orange). Finally, human actions (blue) change the system from the outside by acting directly on the organisms or indirectly by changing abiotic factors. This (non-exhaustive) illustration shows how studies in an agricultural setting can quickly become very complex when the various human actions in agriculture are taken into account.

Soil biodiversity is driven by a number of factors, like e.g. temperature, aridity, soil properties and vegetation types (Delgado-Baquerizo et al. 2018), but also by macro-ecological changes, e.g. invasion of new species (Gornish et al. 2020). Such changes in the composition of the microbial community show its adaptability to new conditions. Human use of land also influences soil life in various ways. Many common agricultural practices alter soil organism communities, which in turn can have an impact on agricultural production (Figure 1). How these changes in the course of agricultural use relate to natural dynamics regarding their extent and reversibility is a question of highest relevance (Bevivino & Dalmastri 2017). At least for some of those practices, changes to the soil organisms are done neither knowingly nor willingly. It is therefore not surprising that human interventions in the context of agricultural use can sometimes have negative impacts on soil health

and the sustainability of farming systems (Yang et al. 2020a). In 2019, more than half (50.7%) of the area of Germany was used for agriculture (OR 4: Statistisches Bundesamt, 2020). This number underlines the importance of sustainable agricultural production, since it is impossible to replace these areas. Nevertheless, knowledge is only just beginning to develop about how the different agricultural uses and practices affect soil microbial life, and how these effects can perhaps even be exploited in a positive way (Głodowska & Wozniak 2019; Alaoui et al. 2020; Yang et al. 2021). As mentioned above, agricultural areas are subject to a variety of human influences. One of the most fundamental of these is the widespread and intensive use of fertilisers. Different fertilisation regimes lead to alterations in soil pH, microbial community composition, transformation and decomposition of organic material, and greenhouse gas emissions (Beauregard et al. 2009). At the same time, agriculture worldwide strongly depends on the use of mineral fertilisers. In fact, it has been estimated that about half the world's population is dependent on nitrogen fertilisers produced using the Haber-Bosch process (Erisman et al. 2008). As the Haber-Bosch process consumes large amounts of energy and mineral fertilisation is associated with environmental damage, organic farming without the use of mineral fertilisation is often considered as an alternative. It is debatable whether organic farming can be productive and profitable enough to feed the world's population (Badgley et al. 2007; Connor 2008; Lakner et al. 2017). As a consequence, agricultural production will depend on mineral fertilisation for the time being.

With this necessity in mind, it is significant to recognise and understand in detail the implications of fertilisation with mineral nitrogen being practised on such a large scale. The use of mineral fertilisers over decades is changing the (bio-)chemical characteristics of our farmland in the long term. These permanent effects of mineral fertilisation on the soil can be attributed to a large extent to soil acidification (Barak et al. 1997; Dal Molin et al. 2020). Since acidified soil has manifold negative impacts on the productivity and sustainability of agricultural land as well as causing environmental damages, this effect of mineral fertilisation needs to be mitigated in some way (Goulding 2016). Mitigating acidification and its effects on the soil ecosystem is traditionally performed by directly counteracting on soil pH. Besides liming with mineral calcium carbonate, similar effects can be achieved using organic soil amendments (Naramabuye & Haynes 2006). This is because of several characteristics these materials may have. For one, composts and manures contain carboxylic, phenolic and enolic groups associated with humic material and can thus consume protons from soil (Wong et al. 1998). Similarly, simple organic anions can consume protons at low soil pH (Sparling et al. 1999). Lastly, manures themselves often contain a considerable amount of calcium carbonate that had been provided to the livestock (Mokolobate & Haynes 2002). In addition to the liming effect, amending soil with organic materials has been shown to increase soil organic carbon and nitrogen contents (Paustian et al. 1992; Xie et al. 2021). Apart from abiotic soil factors, soil-dwelling organisms are also influenced by the addition of

mineral fertilisers. Inorganic nitrogen fertilisation caused a decline in microbial respiration irrespective of the type of inorganic nitrogen source used (Ramirez et al. 2010a). The diversity of soil communities and their species composition are strongly driven by the fertilisation regime (Zhong et al. 2015). For archaea, bacteria and fungi, however, these effects are different (Li et al. 2019a). Focussing on bacteria, Ramirez et al. (2010b) demonstrated in a comparative study that while nitrogen fertilisation leads to consistent changes in the composition of soil communities, effects on their overall α-diversity are site-specific. This regularity suggests that, even in different soil ecosystems, similar groups of bacteria are either at an advantage or at a disadvantage in fertilised soils. Accordingly, nitrogen metabolism pathways such as denitrification, assimilatory nitrate reduction or the organic nitrogen metabolism were found increased in bacteria (Li et al. 2019a).

Related to the topic of fertilisation, but not identical, is the improvement of arable soils with the help of organic substances. Soil improvement is usually not practised alone – especially in conventional agricultural systems of industrialised countries – but in combination with mineral fertilisation. Manure from livestock, crop residues and compost additions are among the most commonly applied. As mentioned above, these substances can counteract acidification caused by mineral fertilisation by buffering the soil pH value. The effects on crop productivity seem to depend on the type of organic material used, with manure increasing yield the most (Paustian et al. 1992; Kätterer et al. 2014). However, meta-analysis shows that the effects of organic inputs on crop yields depend on the exact circumstances and are low in general (Hijbeek et al. 2017). Therefore, organic inputs should not generally be regarded as fertiliser. Rather, their use is intended to improve the sustainability and thus the long-term productivity of agroecosystems (Li et al. 2015; Zhao et al. 2016). Hence, organic soil amendments might help to achieve the long-term goals in agricultural production, while not showing immediate effects in all cases.

Besides abiotic effects, e.g. an increased water holding capacity (Hudson 1994; Anik et al. 2017; Edeh et al. 2020), manifold effects of organic soil amendments on the microbial soil community have been demonstrated in the past. Since a rich and active microbial soil community is thought to be key for a sustainable land management (Wagg et al. 2014), understanding how organic materials impact on the soil ecosystem is an important prerequisite for the development of novel soil management strategies. It has been repeatedly shown that soil amendment with different kinds of organic material has positive effects on the soil microbial diversity and activity when compared to mineral-only fertilisation practices. For example, proteolytic enzyme activities were found enriched in soils amended with a different organic amendments. These findings were reflected by the proteolytic bacterial soil community (Sakurai et al. 2007; Jatana et al. 2020). Soil organic carbon content, soil enzyme activities and the microbial community composition have been shown to correlate with application of amendments (Amadou et al. 2020). Especially manure proved to be

an efficient agent to maintain a high species and functional diversity in soil under agricultural conditions (Li et al. 2020; Luan et al. 2020; Tang et al. 2020; Zhu et al. 2020).

These effects on microbial diversity microbial communities last for several months and can be maintained for years through repeated applications (Montiel-Rozas et al. 2018; Sadet-Bourgeteau et al. 2018). However, the exact manifestation in the microbial community depends on the soil type (Sadet-Bourgeteau et al. 2019). In addition, long-term consistency of soil management is necessary for high microbial diversity (van der Bom et al. 2018). These observations raise the question of the mechanisms by which organic inputs affect microbial diversity. One likely explanation is the structural and chemical heterogeneity that results from the introduction of these substances into the soil ecosystem. For example, application of organic inputs results in increased macro-aggregates, which in turn provide anaerobic habitats (Zhang et al. 2015; Zou et al. 2018). With this increasing complexity, microbial diversity can thus also increase. But also the degradation of organic matter itself promotes biodiversity through the need for more complex degradation pathways represented by microbial consortia. This is also reflected in more complex interactions between the dissolved organic matter and the bacterial community (Li et al. 2019b).

The effects of enhanced heterogeneity in the soil environment can be seen in a number of results from literature. Generally, increases in both soil organic carbon and total nitrogen contents have been identified as factors of influence for bacterial communities (Li et al. 2017). Specifically, nitrogen supplies from manures have been shown to enhance microbial soil diversity and activity (Chaudhary et al. 2015). Microbial biomass carbon and microbial activity increased more strongly when organic amendments were included in the land management as compared to mineral inputs alone (Ma et al. 2020). More in detail, the structure of the soil microbial community was shown to change when organic material is used as part of the management strategy, possibly even towards a structure that is more similar to natural ecosystems (García-Orenes et al. 2013). Interestingly, differences in soil communities between conventionally managed and organic farming systems have been attributed largely to the use of manure (Hartmann et al. 2015). This observation might be explained by the potential of manures to prevent the loss of bacterial diversity due to mineral fertiliser application and, as demonstrated by Sun et al. (2015a), might further be based on the liming effect of manures on soil. On a functional level, a mixed application of mineral NPK fertilisers and livestock manure increased the abundance of nitrogen cycling genes *nifH*, *nirS*, *nirK*, *nosZ* and, especially, bacterial and archaeal *amoA* (Sun et al. 2015b). In sum, agricultural systems have been shown to benefit from amending with organic materials of several kinds by increased soil fertility, soil health and sustainability (Diacono & Montemurro 2010; Bonilla et al. 2012). These positive effects for agroecosystems are largely mediated by the microbial soil communities that result from specialised land management strategies. Due to their potentially large impact on

agricultural soils, organic soil amendments were proposed as tools to shape the microbial soil communities (Aparna et al. 2014).

1.3 Combined effects of agricultural practices

Investigating individual factors and their influence on soil ecosystems is relatively easy to perform experimentally. In agriculture in particular, however, the human factors of influence are manifold. This multitude makes assessing the respective impacts much more complex (Rillig et al. 2021). On the one hand, the number of factors to be investigated creates experimental challenges. On the other hand, different factors can of course influence each other and thus contribute to a complex overall picture. This also means that an evaluation of certain agricultural practices under otherwise identical circumstances has only limited significance. Synergistic effects have been shown for combinations of practices, which need to be identified and exploited in practice (Alaoui et al. 2020). An example of this is the combination of different factors of influence impacting on the organic carbon content of arable soils (Zhao et al. 2013). For pesticides, cross-effects both among each other and with the application of green compost have been demonstrated in the degradation of herbicides in soils (García-Delgado et al. 2018). However, irrigation techniques and other organic improvement measures can also impact on pesticide degradation (García-Delgado et al. 2019). Conversely, the current state of the soil can modulate the effect of certain management practices. For example, the soil organic matter content has been shown to react differently to management depending on the current size distribution of soil aggregates (Trivedi et al. 2017). All these observations are the result of an interplay of abiotic factors and their interactions with the soil microbial community.

The combination of influencing factors that defines a particular land-use type thus manifests itself via abiotic factors in the microbial communities. Accordingly, different land-use types produce different soil microbial communities (Tian et al. 2017). However, the multitude of possible external and internal factors makes it practically impossible to predict in detail the effects of certain land uses at a given site. One useful way to understand the impact of agriculture on soil organisms might therefore be to compare entire land-use systems. Based on these systems and the differences between them, mechanisms could be identified that might explain the changes in microbial communities. To give an example, one such comparison has frequently been done between organic and conventional farming systems. It has been shown that organic farming worldwide increases the total abundance as well as the activity of soil microorganisms compared to conventional farming (Lori et al. 2017). However, there may be variations depending on the land use, the plant life cycle and the climate zone. Furthermore, organic farming increases the diversity and heterogeneity of soil communities (Lupatini et al. 2017). In most cases, these effects on soil life come at the cost of harvest losses. However, especially in depleted farmland, productivity can

be significantly increased by a more ecologically motivated form of management (Cerecetto et al. 2021). This sustainability aspect results from positive influences on biodiversity, heterogeneity and the activity of soil microorganisms and represents an independent value alongside the productivity of the site. The increased sustainability of production seems to be partly due to a stimulation of plant growth-promoting rhizobacteria (PGPRs) (Cerecetto et al. 2021). In general, organic management practices seem to influence bacteria of the phyla Bacteroidetes and Planctomycetes (Fernandez et al. 2020). Similar to these results comparing conventional with organic farming systems, comparisons between varieties of farming systems can reveal valuable insights into their differential impact on microbial communities.

In summary, individual land management practices affect the soil microbial community. However, as each practice is part of a more complex agricultural use system, their respective effects are sometimes difficult to predict. As mentioned before, the use history of a site has an even bigger influence on the respective microbial assemblage and its reactions to external influences. Under certain circumstances, the history of use can still have an impact on microbial conditions even after more than 100 years (Fichtner et al. 2014), which is particularly important in the cultural landscapes of Europe. Studying the effects of land use changes is therefore relevant in that ecological continuity plays a crucial role in the functioning of soil ecosystems (Fichtner et al. 2014). If one observes the changes in the microbial community after a land-use change, it should be possible to draw conclusions about the direct mechanisms that cause these community changes. Conversely, the changes in the microbial community could provide clues for assessing the sustainability of management and soil health in general.

Efficient ways to capture the biological properties and processes in soils represent the 'new frontier' in measuring soil health (Karlen et al. 2019). DNA-based microbial markers are a promising tool for this purpose (Schloter et al. 2018). When monitoring agricultural land and the impact of agricultural practices on various soil parameters, DNA-based methods promise wide applications (Hermans et al. 2020). A combination of favourable properties makes microbial community analyses well suited for capturing the current state of soils as well as for analysing temporal dynamics (Fierer et al. 2021). However, the development of such applications is still in the early stages and requires further basic research to ensure a reliable basis for the interpretation of the data obtained. For example, the processes occurring when microbial communities undergo a shift due to changes in land use are still not fully understood. Appropriate study systems are therefore essential to draw conclusions on how human interventions in the soil ecosystem influence soil community dynamics. One such study system is long-term agroecological experiments in which different forms of cultivation are compared with each other over decades. Such trials are a source for a variety of scientific purposes and are a valuable tool especially for soil ecology issues (Silva & Tchamitchian 2018). Ultimately, it is only through long-term

experiments that well-founded statements can be made about the long-term effects and sustainability of agricultural measures (Johnston & Poulton 2018).

Accordingly, a central part of this doctoral thesis is devoted to studying the long-term effects of mineral fertilisation in combination with organic soil improvement measures as examples of human impact on the bacterial community of agricultural fields. These studies were carried out on the plots of a long-term trial that had been operated for more than three decades. For the investigation of short-term effects on the microbial community in the context of land-use changes, sites that can be easily influenced and have a well-documented history of use are suitable. Restored opencast mining areas, for example, are well suited for this purpose, as their history offers sites of different ages with comparable initial conditions (cf. Box 1). In addition, agricultural restoration inherently involves a repeated change of use. Thus, these sites offer the possibility of tracing well-documented changes in use and their influence on the respective soil life with the help of a chronosequence approach. In addition, these locations are readily available and can be compared with similar locations worldwide. For these reasons, a chronosequence of former opencast mining sites serves as the object of investigation for the second part of my doctoral thesis. Here, in addition to the microbial dynamics resulting from the ageing of the substrate, I investigate the effects of the changes in land use during the restoration measures.

1.4 Aims of this thesis

The general theme of my work is the analysis of the short- and long-term effects of land-use changes in agriculture on the bacterial soil community of arable land and the subsequent assessment of possible consequences for sustainable use. With a chronosequence of reclaimed post-mining sites as a model system, I investigate the consequences of human action during restoration management on the soil bacterial community of the sites. In collaboration with scientists from other fields of soil research, a comprehensive picture of the processes occurring in the analysed time frame could be achieved. Following developmental stages of more than five decades, I trace the shifts in the bacterial community and compare them to the restoration management steps as well as to the variations in soil physicochemical properties. I aim to disentangle the changes happening due to weathering of the soil material from those caused by human action during restoration.

Box 1: Opencast mines as study systems

While the restoration of opencast pits is a specialised challenge in itself, those sites offer unique research opportunities for understanding the influence of human action on microbial soil life. For comparative studies, it is important to have a minimum amount of similar sites available. Opencast mining is performed all over the world in different ecosystems and climates. In Germany alone, there are to date three active large lignite mining areas with different conditions for reclamation. These are the Rhineland, the Central German and the Lusatian lignite mining areas, of which the Rhineland area is the largest in terms of lignite delivery rate as well as future authorised extraction volume (OR 1: DEBRIV 2020a). In each of those areas, several active mines are available for replication of studies. Due to legal requirements in most cases, the history of restoration, i.e. the documentation of which sites have been reclaimed at which time using which procedures, is of high quality. In Germany, this is regulated by the Federal Mining Act (BBergG), its associated regulations and remediation plans initiated by the federal states (OR 2: DEBRIV 2020b).

This enables researchers to trace the entire development of the soil – at least up to a point when the sites are passed back into private hands. Within a series of reclaimed sites, usually well comparable starting conditions can be assumed. This is true for the properties of the soil material, climatic conditions and in part for the use history of the land including the restoration procedures. The procedures used to restore the land are commonly constant over decades and thus ensure a comparable treatment of sites for later studies. A further benefit of reclaimed mining sites for soil research is the availability of different endpoints for restoration.

Depending on how the mined land was used before exploitation, the reclaimed sites are restored towards cropland, forest, lakes, or even near-natural areas meant to support regional nature conservation efforts. A short information video by RWE Power AG reflects the operators' demands for the reclamation of the former opencast mining areas (OR 3: RWE 2019). This leaves several opportunities for comparative research. Lastly, due to the characteristics of the soil material used during restoration, the soil characteristics change quickly in the beginning and were shown to not reach a steady state even after several decades have passed (Insam & Domsch 1988). This causes a continuous pressure on the microbial communities to adapt and thus will change microbial parameters over time. Such developing systems should be susceptible to human intervention leading to different trajectories in their development. Similar observations were already made in the context of soil development during primary successions (Knelman et al. 2014). Taken together, their good availability, high-quality documentation, comparability within each chronosequence, different restoration endpoints and susceptibility to external factors all make reclaimed opencast mines well-suited study objects for understanding the impact of human actions on soil microbial systems.

The main objective of this project is to find out which members of the soil bacterial community are affected by changes in the soil substrate as a result of mining and subsequent restoration measures, and later by the change from one form of agricultural use to another as restoration progresses. In doing so, the extent of the change within the community and thus its importance relative to other processes is estimated. In addition, a closer look at ecologically relevant groups of bacteria should provide an assessment of how the changes in the bacterial community might affect the sustainability of agricultural management. By this, I investigate the hypothesis **(H1)** that the changes in the bacterial community caused by changes in land use are (1) of a significant magnitude, (2) have a long-term effect and (3) may have implications for the sustainability of land use by affecting bacterial keystone groups or bacteria involved in plant-microbe interactions.

In a second study, I take advantage of a long-term agricultural fertilisation trial to study the long-term impact of different land management regimes on the bacterial community as an example of human action. With a well-documented history of more than three decades, the trial offered established cropping systems for comparisons of two types of organic inputs at different levels of mineral nitrogen supply. In addition to data on physico-chemical soil development, yield data and detailed records of soil treatments are available. Besides differences in bacterial community composition, I assess the impact of the different management combinations on microbial interactions as modelled by network analyses based on data from four seasons. Here, my aim is to study the effects of the applied organic improvement measures on soil bacteria and to identify possible consequences for the productivity and sustainability of the respective forms of land use. The objectives of this second study are, firstly, to identify characterising bacterial species of each treatment, to present possible causes for this observation and to estimate their impact on agricultural use. Secondly, to use comparative network analysis to create a model of interactions among bacterial species to gain insight into the metabolic capabilities of the community in question. This serves to evaluate hypothesis **(H2)** that with an appropriate choice of land management tools, changes in the soil bacterial community can be used to enhance the sustainability of a particular form of land use. Taken together, these experiments should deliver a better understanding of the processes occurring in soil triggered by human actions. Also, they provide starting points for further research regarding the choice of sustainable and ecologically sound land management possibilities by revealing basic concepts of how bacterial communities are influenced by land management.

2 Materials and methods

2.1 Description of the study sites

In the context of this doctoral thesis two experiments of different kinds are investigated. The first deals with soil development after the restoration of opencast mining areas (manuscript **M1**). The development of these soils over a period of more than five decades is investigated and correlated with human interventions during this period. In the second experiment, different agricultural land use forms are analysed in a long-term experiment. These forms of use are then investigated for their long-term impact on bacterial soil communities (manuscript **M2**).

2.1.1 Post-mining site chronosequence

2.1.1.1 Site description

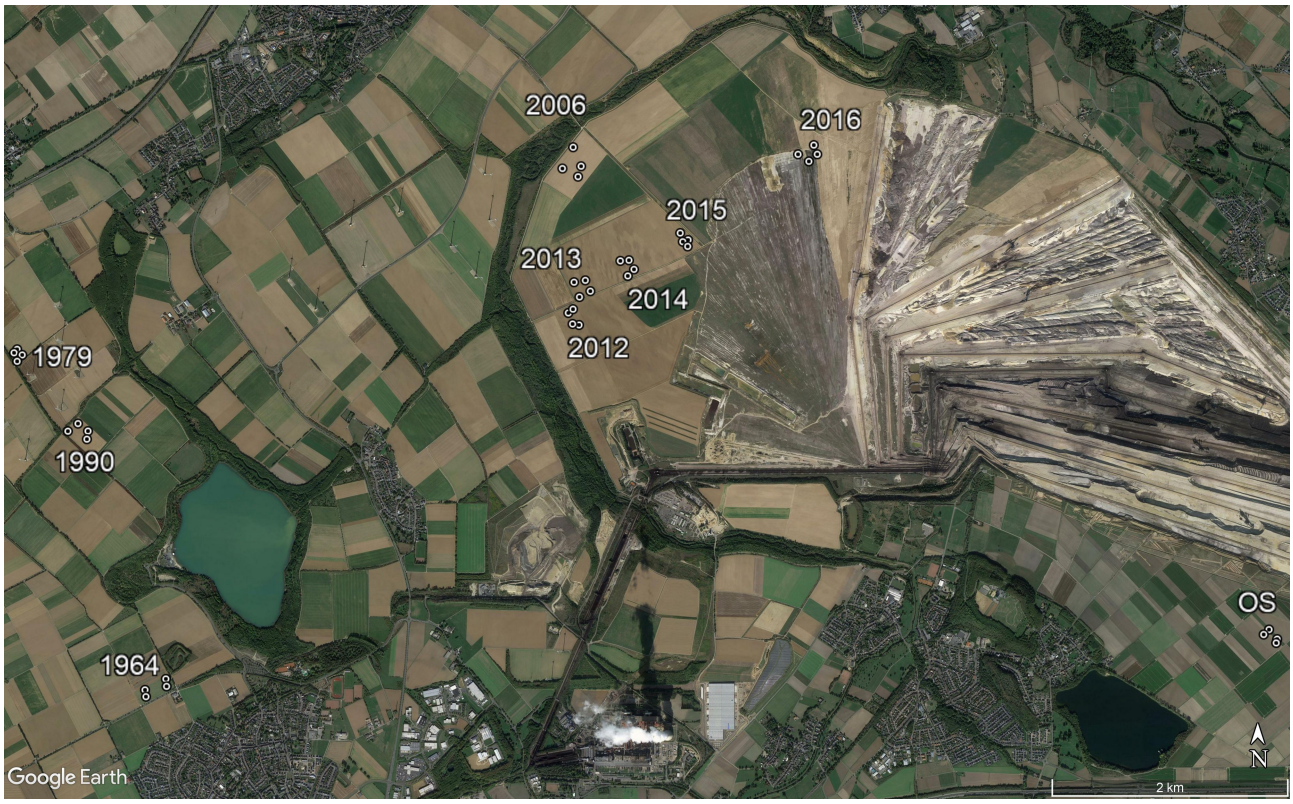

Figure 2: *Overview of the sampling sites near the opencast mine Inden, near Jülich, Germany.*

Each plot was sampled twice, in March and June, in four replicates except for those plots marked 2015 and 2016, which were only accessible in June. OS represents the unmined reference plots. Image material: Google Earth, DigitalGlobe, 2018-05-05, reproduced from ***M1***

The restoration chronosequence consisted of former opencast mining sites of the lignite mine Inden, south of the city of Jülich in Germany (Figure 2; 50° 52′ 46″ N, 6° 19′ 21″ E). In the area of Jülich for the period between 1961 and 2020, the mean annual temperature was 10.1 °C, the

mean relative humidity was 78%, the mean annual sunshine duration was 1579 h and the mean annual precipitation was 692 mm (OR 5: FZ Jülich). During mining, the top soil (Luvisol) and the underlying loess material were removed up to a depth of 6 m. The materials of these layers were then mixed in a ratio of 1:5 and transported to the opposite side of the mine, where restoration takes place simultaneously to the other mining operations. Once there, the mixed soil material is first loosely piled up before it is levelled and compacted after some weeks. This process of soil removal on one side and restoration on the other side of the open pit has been taking place continuously since 1957. In this way, a mosaic of sites of different ages and at different stages of development has been created. However, as these sites all originate from well comparable source materials, they are well suited for comparative studies of different ages. All sites that were investigated in this thesis had been restored to farmland.

The restoration of the exploited areas can be divided into three phases. The first two phases were carried out by the operating company RWE Power AG, and in the third phase the land was finally handed back into private hands. The first phase served to build up organic matter in the soil and to generate soil structure, thus ultimately increasing soil fertility for subsequent agricultural use. For this purpose, alfalfa (*Medicago sativa* L.) was cultivated continuously for three years after a three-month recovery phase during which a soil profile of about 2 m thickness was formed. In addition, the cultivation of alfalfa during this early phase of restoration should stimulate biological activity in the young soils. At the end of this three-year initial phase, the alfalfa fields were tilled in autumn and a layer of compost, but neither mineral fertilisers nor biocides, were applied. In the second phase the land was already available for agricultural use. A crop rotation of winter wheat (*Triticum aestivum* L.), winter barley (*Hordeum vulgare* L.), rapeseed (*Brassica napus* L.) and sugar beet (*Beta vulgaris* L.) was established. For this purpose, mineral fertilisers were applied per hectare and year in a range of 180-210 kg N, 45-100 kg P, 45-130 kg K, and 0-20 kg Mg. Seven years after the restoration of the land, phase two ended with the transfer of the land to local farmers. During phase three, the land continued to be cultivated with the usual regional crop rotation, with a focus on sugar beet cultivation. Detailed information on the use of fertilisers and pesticides during this last phase were not available.

2.1.1.2 Sampling procedures

Nine sites were selected for sampling along a chronological sequence of restored areas. Care was taken to ensure that all the phases described above were recorded. In total, the chronosequence covered the time span from the first storage of the substrate until 52 years after restoration. To this end, two samplings were carried out, one in March and one in June. From phase 1, the freshly deposited substrate and the restoration years 2015, 2014 and 2013 were sampled. However, the fresh substrate and the site of 2015 were only accessible for sampling in June. From phase two one field, which was restored in 2012, was sampled. From the third phase, sites from 2006, 1990,

1979 and 1964 were selected. Finally, samples were taken from a reference plot in the same way. This area was located in front of the mine excavation site and represented the original state of the soils from the region. The close proximity of the reference plot to the excavation site ensured that its properties were similar to those of the topsoil used for mixing the restoration substrate of the chronosequence plots. With the exception of the plots from the earliest restoration phase, care was taken during selection that winter wheat was planted in all fields (including the reference fields) during sampling. This was to avoid an influence of different field crops on the results of the soil microbial analysis. For the early restoration phase, on the other hand, the different vegetation was considered part of the examined restoration process. The average size of the sampled fields was 6 ha. A total of four samples were taken from each of these fields at an average distance of 115 ± 32 m (mean ± SD).

Each sample was mixed from five subsamples taken within a five-metre radius of a recorded GPS position, each subsample consisting of 500 cm^3 of soil from 0-10 cm depth. After mixing, about 10 g of soil were immediately sieved with sterilised tools for microbial analysis (mesh size 2 mm) and stored on dry ice for transport, and later at -80 °C. The remaining sample was kept at 4 °C for physicochemical analysis. The results of the physico-chemical analyses were previously published by Roy et al. (2017) and in the supplement of manuscript **M1**. In the following, these results are briefly summarised for reference. The soil structure of all samples showed similar characteristics, with an average of 17% clay, 78% silt and 5% sand. The lowest value for soil density came from the recently deposited material (1.2 g cm^{-3}), the highest from the reference field sites (1.4 g cm^{-3}). While the soil of the reference plot was slightly acidic and free of calcium carbonate ($CaCO_3$), the restored sites were buffered at alkaline pH values, especially in early years, due to high $CaCO_3$ contents. It was not until about ten years after the restoration that the $CaCO_3$ content and thus also the pH values decreased. At the same time, the content of organic carbon (C_{org}) increased. A particular increase was recorded after the fourth year after restoration, when agricultural use began. In late restored soils, the content of organic carbon was found to be considerably higher than at previous times. A similar development could be observed for the total nitrogen content, especially in the second phase of the restoration process. However, both the values for organic carbon and total nitrogen continued to be lower compared to the reference plot. In contrast, plant-available P increased steadily to values comparable to those of the reference, while the total phosphorus content remained below those of the reference despite an increase over time. The ammonium and nitrate contents were generally low in restored plots. However, elevated levels were recorded in the plots from 1990 and 2006.

2.1.2 Long-term fertilisation trial

2.1.2.1 Site description

The long-term experimental plots were part of the International Organic Nitrogen Fertilisation Trial (IOSDV) at Rinkenbergerhof in Speyer, Germany (Figure 3). Since 1983, the long-term effects of soil improvement measures with organic substances in a gradient of mineral fertilisation had been studied there (Bischoff & Emmerling 1997; Armbruster et al. 2012). The climate at the experimental site had an average annual temperature of 10.0 °C with an average annual precipitation total of 593 mm and sunshine duration of 1,441 h. The soils at Rinkenbergerhof experimental farm before treatments had a pH of 6–6.5, contained on average 8.9% clay, 48.3% silt and 42.8% sand and were classified as Cambisol. Horse manure or crop residues were applied as organic materials to experimental plots of 7.5 x 6 m in size. A third row of plots received no organic matter input as a control. In the manure treatment, 300 dt FM ha-1 of stored horse manure is incorporated into the soil every third year after winter barley cultivation. The plots treated with plant residues receive the harvest residues of the respective field crop (cereal straw or sugar beet leaves) as well as the chopped catch crops. In 2015, this corresponded to 10.2 dt ha-1 of barley straw. The main difference between these treatments was therefore the use of animal manure for soil improvement as opposed to improvement measures with plant biomass. Mineral fertiliser was applied in five different levels between 0 and 240 kg ha-1 (or 200 for barley). The combinations of organic soil improvement and mineral fertiliser levels were each replicated three times in the IOSDV. All plots investigated were ploughed equally to a depth of 30 cm. This was done once between the first and second sampling during the study period (see below for

Figure 3: *Aerial images of the field experiment*

Aerial images of the Rinkenbergerhof experimental farm in Speyer, Germany (top) and the IOSDV (bottom). In addition to the ploughed plots with 1983 as the year of establishment, there have also been experimental plots with reduced tillage and no-tillage since 2005. Image material: Google Earth, Landsat / Copernicus, 2020-04-26

sampling times). In addition to the above-mentioned fertilisation and soil improvement measures, all plots were supplied with a basic fertilisation of a total of 35 kg P ha-1, 232 kg K ha-1, 66 kg Mg ha-1 and 1.6 kg Mn ha-1 in spring (P, K Mg) and autumn (Mn). The crop rotation over the years was sugar beet, winter wheat and winter barley. *Raphanus sativus* var. *oleiformis* was used as a catch crop after winter barley in the plant-improved plots. The catch crop was mulched before ploughing.

2.1.2.2 Sampling procedures

From the available experimental plots, 18 were selected for this doctoral thesis, each of which was sampled four times over the seasons. Of the five levels of mineral fertilisation, two were selected, namely 'without' (0 kg N ha-1) and 'with' (120 + 60 kg N ha-1 to sugar beet, 90 + 50 + 40 kg N ha-1 to winter wheat and 80 + 30 + 40 kg N ha-1 to winter barley) mineral fertilisation. These were studied in all organic soil amendment variants and all three replicates. This resulted in a total number of 72 samples when the repeated sampling was included. The sampling campaigns covered the complete cultivation period of sugar beet including soil preparation starting in September 2015 with fallow soil with volunteer or catch crop seedlings before the application of manure. After tillage and incorporation of the catch crops, the winter sampling took place in February 2016. The next samples were taken in June after sowing of sugar beet in April and two applications of mineral fertiliser in May. The final sampling then took place shortly before the harvest of the field crop in November. The variation of each plot was captured with twelve punctures using a core sampler. The sampled material was then thoroughly mixed by hand. In line with the procedure for manuscript M1, a sub-sample of approximately 100 g was sieved directly on site (2 mm) and stored on dry ice for transport. The remaining material was stored at 4 °C and used for physicochemical analyses according to VDLUFA (1991). The replicates of the experiment were considered true replicates for the evaluations.

The results of the physicochemical analyses were presented in the supplementary material of manuscript M2. As these values form the basis for the following considerations, they are briefly summarised here together with data collected and provided by LUFA Speyer. Over the course of the trial, the soil pH value fell in those plots that received mineral fertiliser, in some cases significantly, to 4.7 – 6.2. Plots that did not receive any organic matter recorded the greatest changes. In contrast, plots that did not receive any mineral fertiliser remained at 6.0 – 6.8, comparable to the initial condition. Overall, the total nitrogen content varied from 0.06% to 0.1% and the total carbon content from 0.5% to 1.0%. In the control plots, which did not receive any organic matter, the above-mentioned management measures led to a reduction in the organic carbon content. It dropped from the original 0.83% to 0.60–0.70% in the upper soil layer of 0–30 cm. In contrast, both variants with addition of organic matter remained close to the initial value with 0.74–0.85% for manure and 0.74–0.82% for crop residues. Similarly, organic nitrogen content in

unamended soils decreased from 0.07% to 0.053-0.065%. Here, too, only slight changes were visible in the improved soils. Soils with horse manure were at 0.066-0.077% organic nitrogen content and soils with plant residues at 0.068-0.076%. Ammonium levels did not differ in any of the treatments and were overall at a low level. As could be expected, nitrate levels differed mainly between treatments with and without the addition of mineral nitrogen with plots receiving mineral N also exhibiting higher contents of nitrate. Total potassium was consistently higher in plots where organic amendments were applied compared to control, but not between amendment types. Total phosphorus, on the other hand, differed only between plots with and without mineral fertilisation, but not between organic amendment treatments.

2.2 Sample processing for bacterial community analyses

2.2.1 Soil DNA extraction and 16S rRNA gene quantification

After preparatory tests, the total DNA of the samples of each experiment was obtained with the most appropriate extraction kit. For the soils of the restoration chronosequence (manuscript **M1**), the PowerSoil DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, CA, USA) provided the best results. Due to the special composition with high amounts of $CaCO_3$ in the young soils, the DNA quality of other tested extraction kits was not sufficient for the following steps. DNA was extracted from 0.25 g fresh weight soil sample from a total of 76 samples (ten developmental stages incl. control plots, see above; two sampling time points – two stages only available at one time point; four pseudo-replicates per stage and time point; four negative controls) according to the manufacturer's instructions. For the long-term experiment at LUFA Speyer (manuscript **M2**), the NucleoSpin Soil Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) was used for DNA extraction. All 72 field samples as well as a negative control for each time point were processed. The manufacturer's protocol provided for extraction from 0.5 g fresh weight soil. Extracted DNA was subjected to a quality control checking DNA concentrations as well as integrity using either (manuscript **M1**) a fragment analyzer (Advanced Analytical Technologies, Inc., Ames, US-IA) or (manuscript **M2**) a Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, US-MA) and visual inspection on a 1% agarose gel.

To document the development of the abundance of bacteria in the soils of the chronosequence (manuscript **M1**), the abundance of the 16S rRNA gene was assessed using quantitative real-time PCR. For this purpose, preliminary tests were carried out to determine an optimal dilution with which the PCR reactions ran most efficiently. Subsequently, the samples were measured at a dilution of 1:16 using the 2x Takyon for SYBR Assay master mix (Kaneka Eurogentec S.A., Seraing, Belgium) according to the manufacturer's instructions and an additional 0.06% Bovine serum albumin (Sigma-Aldrich Corp., St. Louis, MO, USA). Primers were FP 16S rDNA (5′-GGTAGTCYAYGCMSTAAACG-3′) and RP 16S rDNA (5′-GACARCCATGCASCACCTG-3′) as

described by Bach et al. (2002). Besides the actual samples, each reaction plate carried a standard curve as well as three negative PCR reactions. The PCR program involved an initial 3 min denaturation step, followed by 40 amplification cycles (denaturation 10 s, 95 °C; annealing 20 s, 58 °C; elongation 45 s, 72 °C) and was carried out on a 7300 realtime PCR System (Applied Biosystems, Foster City, US-CA). Finally, a melting curve analysis was performed for each reaction plate to confirm the specificity of the reactions. The real-time quantitative data were then analysed in R with the package qpcR (Spiess 2014) using the mechanistic cm3 model (Carr & Moore 2012). Outliers with different reaction kinetics were identified and removed and a baseline subtraction and normalisation performed. This model is robust to PCR inhibitions and thus low PCR efficiencies. After calculation of initial fluorescence values per sample, these were converted to gene copy numbers per gram of dry soil using standard curves ($R^2 > 0.99$ for all plates) from 10-fold dilutions of standards (16S rRNA gene of *Pseudomonas putida*, $5.6 \times 10^4 – 5.6 \times 10^8$ copies / µl).

2.2.2 Amplicon library preparation and sequencing

Amplicon libraries were prepared by PCR from 10 ng input DNA. The V1-V2 region of the bacterial 16S rRNA gene was amplified. For this purpose, the primer pair S-D-Bact-0008-a-S-16 (5′-AGAGTTGATCMTGGC-3′) and SD-Bact-0343-a-A-15 (5′-CTGCTGCCTYCCGTA-3′) as described by Klindworth et al. (2013) and the NEBNext High-Fidelity 2x PCR Master Mix (New England Biolabs, Ipswich, MA, USA) was used to prepare the reactions. To compensate for random variation between reactions, all PCRs were performed in three technical replicates. Negative extractions were included in their own reactions as well as PCR negative controls. The PCR programme consisted of an initial denaturation step of 5 min at 98 °C, 25 cycles of amplification (denaturation 10 s, 98 °C; annealing 30 s, 60 °C; elongation 30 s, 72 °C) and a final elongation step of 5 min at 72 °C. The libraries were then visually inspected on a 1% agarose gel before the technical replicates were pooled and purified using a NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel GmbH & Co. KG). Further quality controls were performed using either (manuscript **M1**) a fragment analyser or (manuscript **M2**) a 2100 Bioanalyzer instrument (Agilent Technologies, Inc., Santa Clara, US-CA), ensuring in each case that the amplicon size was as expected and that no primer dimers were present in the pooled samples.

The pooled and purified amplicon libraries were then indexed for multiplexed short-read sequencing using a Nextera XT Index Kit v2 (Illumina, Inc., San Diego, US-CA). For this purpose, 10 ng of amplicon DNA were subjected to a further PCR step with the respective indexing primers. The PCR protocol for this consisted of an initial denaturation of 30 s at 98 °C followed by eight amplification cycles (denaturation 10 s, 98 °C; annealing 30 s, 55 °C; elongation 30 s, 72 °C) and a final elongation phase of 5 min at 72 °C. The subsequent quality checks were in each case analogous to those after the previous PCR step. Finally, the indexed libraries were diluted to a concentration of 4 nM and were pooled equimolar. Sequencing was performed on an Illumina

MiSeq system (Illumina, Inc.) using 10 pmol DNA including 20% PhiX as positive control according to the manufacturer's instructions. Paired-end sequencing with the MiSeq Reagent Kit v3 (600 cycle) was chosen. The raw sequencing data were demultiplexed by the MiSeq system.

2.2.3 Bioinformatic processing of sequencing data

The subsequent bioinformatic processing of the data differed between manuscripts **M1** and **M2** due to further developments in methods and associated international standards between the studies.

For manuscript **M1**, remaining primer and adapter sequences were identified and removed using the software cutadapt v. 1.14 (Martin 2011). In the following, only the forward reads (300 bp) were further processed. PhiX sequences were filtered from these, a sequencing quality filter was applied, and the individual reads were trimmed by 10 bp from the 5' end and by 30 bp from the 3' end. Afterwards, technically induced sequencing errors of the reads were eliminated with DADA2 v. 1.4.0 (Callahan et al. 2016). DADA2 infers the exact sequence of each PCR amplicon, also called amplicon sequence variant (ASV). Chimeric sequences were removed from the generated ASVs and taxonomic information was assigned based on the SILVA database v. 128 (Quast et al. 2013). Since ASVs can differ even by single nucleotides, it was suspected that the bacterial diversity of the samples could be considerably overestimated. The calculated ASVs were therefore clustered to operational taxonomic units (OTUs) at 97% sequence similarity using MeShClust2 v. 2.3.0 (James & Girgis 2018). The data set prepared in this way was imported into R v. 3.5.1 (R Core Team 2015) and further edited with the package phyloseq v. 1.22.3 (McMurdie & Holmes 2013). There, another 19 OTUs were deleted that were also found in negative controls, as well as OTUs that were assigned to chloroplasts or mitochondria or not to the kingdom of bacteria.

For manuscript **M2**, primers and adapters were removed using AdapterRemoval v 2.1.7 (Lindgreen 2012). PhiX was then removed and forward and reverse reads were merged applying DeconSeq v. 0.4.3 (Schmieder & Edwards 2011). This data was further processed with the QIIME pipeline v. 1.9.1 (Caporaso et al. 2010). To do this, the reads were first filtered based on their Phred quality score at a threshold of three and only reads between 300 and 400 bp were considered further. After the reads were clustered at 97% sequence similarity, taxonomic information was assigned by use of the RDP classifier v. 2.2 (Wang et al. 2007) retrained with the GreenGenes database v. 13.8 (DeSantis et al. 2006). Now, rare OTUs with a relative abundance of 0.005% or less were removed from the dataset. After importing the data into R v. 3.3.2 with phyloseq v. 1.19.1, OTUs that were also found in the negative controls were removed, as were those that had been identified as chloroplast or mitochondrial sequences.

2.3 Statistical analyses

Since the experimental setup differed strongly between the two experiments investigated for this thesis, the statistical methods used to analyse their data had to be adjusted to the respective needs. Thus, the statistical methods are described in the following separately for each experiment.

2.3.1 Analyses of the post-mining site chronosequence data (M1)

Shannon diversity and Pielou's evenness index were calculated from all 8,254 OTUs remaining from previous steps. For further analyses OTUs present in only a single sample were eliminated and the count data of each sample were subsampled to 46,858 reads per sample. Saturated rarefaction plots confirmed a representative number of reads in all samples. Since it was expected that samples would be more alike within the same restoration phase than between phases, a hierarchical clustering was done. For this, the Ward clustering algorithm was applied on Bray-Curtis distances (Murtagh & Legendre 2014). A meaningful clustering result was confirmed by ordination using constrained analysis of principal coordinates (CAP) on Bray-Curtis distances with the cluster affiliations as independent variable. With the R package vegan v. 2.4.5 (Oksanen et al. 2017) the significance of the model and its axes was tested by permutation testing with 20,000 permutations adjusting for repeated samplings and pseudo-replicates.

Bacterial responders to restoration age on the level of phyla were identified by linear mixed models applying the implementation from the R package lme4 v. 1.1.14 and lmerTest v. 3.0.1 for hypothesis testing (Bates et al. 2015; Kuznetsova et al. 2017). The only fixed factor for the models was the restoration age cluster while sampling date was included as a random factor. Normal distribution and homoscedasticity of model errors were both checked graphically in qq- and residual plots. To test specific hypotheses for differences between groups of age clusters, orthogonal planned contrasts were defined. Multiple testing was accounted for on the level of the overall tests using the Bonferroni method. For OTU level analyses a custom subsetting approach was developed. Subsets represented OTUs that were only present in a single age cluster (or the reference plots) or in all restored sites (i.e. a core microbiome of restored post-mining sites). The 20 most abundant of those OTUs that had a taxonomic information at least on order level available were then compared between subsets. This approach proved to deliver equivalent results as an indicator species analysis (De Cáceres & Legendre 2009) and as a random forest analysis. As a benchmark for the restoration age effect, the numbers of OTUs specific for each sampling date was calculated and averaged. The obtained results were further compared to three data sets on similar case studies on other restored open-cast mining sites. Those data sets used comparable sequencing approaches on the Illumina MiSeq platform and are publicly available from the NCBI Sequence Read Archive. The first study comprised sites from West Virginia, USA, until 32 years after restoration (Kane 2019). Data are available under the BioProject ID PRJNA529237. The

second one included sites until 30 years after restoration in Virginia, USA, re-forested with native hardwood trees (Sun et al. 2017). These data are available with ID PRJNA324696. The third data set (PRJNA529248) included soils from until 13 years after restoration from a former mine in Mississippi, USA. All those were processed analogously to the procedures described above.

Statistical comparisons of soil physicochemical parameters were done using robust ANOVAs. For this purpose, parameters measured only once (all total contents, $CaCO_3$ content, C_{org} as well as their ratios) were analysed by robust one-way ANOVA to compare measurements between the previously identified age clusters. This was done using the R function t1wayv2() by Wilcox (2013). All parameters measured twice (pH_{CaCl2}, plant-accessible contents, NH_4^+ and NO_3^- contents) were compared by robust two-way ANOVA with the function bwtrim() disregarding samples from the sites only sampled once. Analogous to the LMMs, planned contrasts were used for testing hypothesis about differences between groups of restoration age clusters. These were calculated using the functions lincon() and bwamcp(). Comparisons of effect sizes were done with the generalised eta squared (η^2) measure (Bakeman 2005).

2.3.2 Analyses of the long-term experimental data (M2)

As in manuscript **M1**, α-diversity and evenness were calculated from the full, unfiltered data set. The 3,282 OTUs remaining after filtering were included in an ordination analysis. As above, a CAP was used with vegan package v. 2.4-1. In order to be able to evaluate only the influences of fertiliser management, those of the soil parameters water content, pH value, ammonium, nitrate, sulphur, phosphorus, potassium and magnesium content, as well as the sampling time, were partialled out in CAP. All of the above-mentioned parameters showed significant influence on the bacterial community of the test soils in an initial analysis step. The statistical significance of the experimental factors mineral fertilisation and organic soil improvement were tested using a permutation test with 999 permutations, taking into account the repeated sampling.

OTUs that were specific to one form of organic soil amendment were identified by means of machine learning. The BioMiCo algorithm with a delay phase of 5,000 steps was used (Shafiei et al. 2015). A share of 67% of the samples was included in the training phase and 33% in the test phase. Multiple re-calculations ensured that the results were robust. The algorithm calculates groups of OTUs that are indicative for the defined treatments from the abundance data. The 20 highest OTUs in terms of posterior probability within a group were further considered as indicative OTUs for their treatment. A visualisation of the results with the help of a heat map served to better interpret the results. A co-occurrence analysis provided a model for reflecting bacterial interactions in the experimental soils. The MetaMIS v. 1.02 software with default settings was chosen for this purpose (Weng et al. 2017). By generating one network per combination of treatments, the effects of each treatment on co-occurrence could be visualised. MetaMIS was provided with the mean

abundance of taxonomic families across all field replicates as input. Finally, for numerical description and statistical comparisons of the resulting networks, the average degree, clustering coefficient and path length of all networks were calculated.

For statistical evaluation of the measurement data, Bayesian methods were preferred over frequentist methods because they offer more flexibility and, moreover, allow valid statements even with low replication numbers. Accordingly, Bayesian models were used for the physicochemical soil parameters, DNA content, α-diversity and abundances of the taxa studied. The models were comparable to linear mixed models and included the sampling time as a random factor. For the evaluations, long-term data on the yields of the respective plots were additionally contributed by the Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) in Speyer, Germany. These were assessed for the period between 2005 and 2016, i.e. four complete crop rotations. A Bayesian two-way ANOVA model was fitted for this purpose comparing yields between the treatment combinations. During the evaluations, it was noticeable that one of the examined plots was an extreme outlier in almost all measurements. This plot from the combination 'mineral fertilised, without organic improvement' was therefore deleted from all analyses. For the Bayesian models, Markov Chain Monte Carlo (MCMC) sampling was used in the software packages JAGS v. 3.2.0 (Plummer 2003) and Stan v. 2.10.0 (Stan Development Team 2015) as well as utilities by Kruschke (2015) to draw representative samples of the posterior distribution. After a burn-in of 2,000 and an adaptation phase of 1,000 steps, a total of 2 million steps were calculated in four independent chains. Every tenth step was recorded. Convergence of the individual chains was visually verified by trace plots to ascertain that all chains came to the same result. The effective sample size (ESS) was used to detect autocorrelation within the chains. A lower limit of 10,000 was chosen to provide a representative sample of the posterior distribution. To test statistical hypotheses, 95% highest density intervals (HDI) of the difference between two means were used. If the HDI did not contain a value of zero, a statistically credible difference between the two means was assumed, as a difference of zero had only a low posterior probability. This procedure was chosen because it is more robust than Bayesian p-values or Bayes factors. It also provides more information for judging the differences. To calculate an HDI, the difference of the mean values at each recorded step of the MCMC chain was taken. Finally, the 95% HDI includes those values with 95% highest posterior probability.

2.4 Brief method descriptions for supporting manuscripts

The manuscripts **M3** and **M4** (s. below) each merely serve as supplementary information for the discussion of the results and are therefore not a main component of this doctoral thesis. The methods used in both publications are therefore abridged below.

2.4.1 Mesocosm experiment on influence of high-carbon amendments

The experimental setup took place in the experimental garden of the University of Lüneburg (Lüneburg, Germany, 53° 14′ 23.8″ N 10° 24′ 45.5″ E, 718 mm mean annual precipitation, 9.2 °C mean annual temperature). Mesocosms with a volume of 38 litres were filled with a Cambic Luvisol from the experimental farm Hohenschulen (Achterwehr, near Kiel, Germany, 54° 16′ 36.80″ N, 9° 58′ 48.0″ E), with a density of about 1.1 g cm^{-3}. The soil had a total carbon content of 1.26%, a total nitrogen content of 0.14%, a C:N ratio of 9.2 and a pH of 6.0 at the onset of the trial. The investigated mesocosms were first planted with spring barley (*Hordeum vulgare* cv. Barke) and faba bean (*Vicia faba* cv. Tiffany) as preceding crops. After their harvest in autumn, winter barley (*Hordeum vulgare* cv. Antonella) was planted in the same mesocosms as the main crop. Planting density, fertiliser regime and pest control were based on common agricultural practices in Germany. The two high-carbon amendments (HCAs) spruce sawdust (C:N 539) and wheat straw (C:N 71) were applied at a rate of 8.6 t ha^{-1} in the period between the preceding crop harvest and the subsequent sowing and mixed with the top 10 cm of the mesocosm soils. In addition, a variant without HCA was established as a control. All plant harvests were done by removing the aboveground plant parts only. Soil samples were collected and pooled after the preceding crop harvest (T1) and after the main crop harvest (T2) in the form of 6 soil cores (diameter 1 cm, length 10 cm) per mesocosm. The preparation of the soil samples, extraction of DNA, generation of sequencing libraries and next generation sequencing were carried out analogously to the procedure described above for manuscript **M1**. QIIME 2 v. 2018.8.0 with the DADA2 plugin v. 1.8.0 served for the bioinformatic preparation of the data (Caporaso et al. 2010; Callahan et al. 2016). ASVs were generated at 99% sequence similarity and taxonomically annotated against the SILVA database v. 132 (Quast et al. 2013).

After processing and removing ASVs from contaminants, 18,834 ASVs remained in the dataset. From these, Shannon diversity and Pielous evenness were calculated and statistically compared between treatments. The effects of crop rotation and preceding crops on the bacterial soil community were determined using PERMANOVA over the entire data set and within the individual sampling dates T1 and T2. Differential abundances of individual ASVs between the different treatments were statistically evaluated using the DESeq2 package v. 1.24.0 using the Benjamini-Hochberg correction (Benjamini & Hochberg 1995). A more detailed version of the materials and methods used can be found in **M3** and from van Duijnen et al. (2018).

2.4.2 Soil incubation study with high-carbon amendments

The soil incubation experiment was carried out with soil from the same experimental farm as **M3** (cf. 2.4.1). Wheat straw, spruce sawdust and lignin were used as HCAs, each of which was added to the soil at a rate of 1.5 g C kg^{-1}. This corresponded to an admixture of 3.4, 3.3 and 2.4

2.4 g DM kg^{-1}, respectively. Of the total amount, 90% was mixed with the soil, and the remaining 10% was buried in nylon nets at a depth of 2 cm. The experimental set-up was carried out in segmented stainless steel tubes of 30 cm height and 20 cm diameter, the six segments of which could be individually filled and removed for analyses. The tubes were sealed gas-tight with a lid that left about 8,000 cm^3 of gas space above the soil surface. Five types of experimental soils were filled into these mesocosms with a density of 1.5 g cm^{-3} and reactivated by adding deionised water. All soils containing HCAs later received an ammonium sulphate solution as nitrogen fertiliser. In addition, there were two control soils without HCAs but with or without later addition of nitrogen fertiliser. The soil moisture was adjusted to 60% of the water holding capacity (WHC) of the soil in all treatments and kept between 50 and 60% WHC for the duration of the experiment. The prepared mixtures were incubated at 20.3 to 22.3 °C for 120 days. Seven days before adding the fertiliser solution, the first soil segment was removed, the others then 7, 21, 49, 77 and 113 days after fertiliser addition. Soil chemical analyses, a determination of the microbial biomass and the abundance of ammonia-oxidising bacteria and archaea as well as various denitrifiers were then carried out. Details on the chemical analyses and the determination of microbial biomass can be found in Reichel et al. (2017).

DNA extraction was carried out with the NucleoSpin Soil Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) analogous to **M2**, the subsequent real-time PCR analyses including data preparation were carried out analogous to those in **M1**. PCR primers for the genes amoA (ammonia monooxygenase), narG (nitrate reductase), nirK / nirS (nitrite reductases) and nosZ (nitrous oxide reductase) were used (Bru et al. 2007; Zhang et al. 2013). The gas-tight experimental setup allowed a gas chromatographic analysis of CO_2 and N_2O emissions from the experimental soil. An electron capture detector and a flame ionisation detector (GC-ECD/FID, Clarus 580, PerkinElmer, Rodgau, Germany) were used. For the statistical analyses, ANOVA with Tukey-B and Games-Howell post-hoc tests for pairwise comparisons were used. More details on the material and methods can be found in **M4** and in Reichel et al. (2017).

3 Overview of publications and contributions

This thesis is based on the following publications:

- **Schmid, C.A.O.**, Reichel, R., Schröder, P., Brüggemann, N. & Schloter, M. (2020) 52 years of ecological restoration following a major disturbance by opencast lignite mining does not reassemble microbiome structures of the original arable soils. *Science of The Total Environment,* 745, 140955. https://doi.org/10.1016/j.scitotenv.2020.140955 **(M1, published)**
- **Schmid, C.A.O.**, Schröder, P., Armbruster, M. & Schloter, M. (2018) Organic amendments in a long-term field trial – consequences for the bulk soil bacterial community as revealed by network analysis. *Microbial Ecology*, 76, 226–239. https://doi.org/10.1007/s00248-017-1110-z **(M2, published)**

Relevant contributions as co-author that are cited but not included in this thesis:

- Kamau, C.W., van Duijnen, R., **Schmid, C.A.O.**, Balázs, H.E., Roy, J., Rillig, M., Schröder, P., Radl, V., Temperton, V.M. & Schloter, M. (2021) Impact of high carbon amendments and pre-crops on soil bacterial communities. Biology and Fertility of Soils, 57, 305–317. https://doi.org/10.1007/s00374-020-01526-0 **(M3, published)**
- Reichel, R., Wei, J., Islam, M.S., **Schmid, C.A.O.**, Wissel, H., Schröder, P., Schloter, M. & Brüggemann, N. (2018) Potential of wheat straw, spruce sawdust, and lignin as high organic carbon soil amendments to improve agricultural nitrogen retention capacity: an incubation study. Frontiers in Plant Science. 9, 900. https://doi.org/10.3389/fpls.2018.00900 **(M4, published)**

3.1 Manuscript descriptions and contributions

3.1.1 Manuscript M1

52 years of ecological restoration following a major disturbance by opencast lignite mining does not reassemble microbiome structures of the original arable soils

Christoph A. O. Schmid, Rüdiger Reichel, Peter Schröder, Nicolas Brüggemann & Michael Schloter

Summary:

The extraction of raw materials in opencast mining processes devastates large areas of land every day anew, which later have to be made usable again. This manuscript traces the developments that the bacterial soil community undergoes over a period of 52 years as a result of the restoration of opencast mining areas. The organisms proved to be influenced both by the restoration process itself and by subsequent weathering processes of the parent material. A comparison with untouched soils near the excavation front allowed an assessment of whether the bacterial soil communities were able to reach a stable state during these 52 years and whether this state resembles the initial state. The results obtained indicate that there were three phases during the restoration period studied, which can be distinguished on the basis of their specific bacterial communities. The differences between these phases were of similar magnitude to the seasonal dynamics measured on the same surfaces. Typical representatives of the early restoration phase were Flavobacteriaceae, Cytophagaceae and Sphingobacteriaceae. In addition, Rhizobiales were found, which were probably promoted by the cultivation of the legume Alfalfa during the first restoration phase. In late years of restoration, Peptostreptococcaceae, Desulfurellaceae and Streptomycetaceae were most prominent. Although both abundance and diversity were back to baseline after five years, an analysis of bacterial community composition showed that even 52 years after restoration, the soils are hardly comparable to the control plots in terms of their microbial parameters. In order to draw more general conclusions, the results were also compared with those of three other former opencast mining areas. Comparable trends were found between the sites despite different restoration strategies. This suggests that similar measures can be used in the restoration of opencast sites with regard to their microbial parameters.

Contributions of C.A.O. Schmid:

- contributed to sampling design and sampling
- performed sample preparation, laboratory analyses, sequencing, data analyses
- wrote the manuscript as lead author

3.1.2 Manuscript M2

Organic amendments in a long-term field trial – consequences for the bulk soil bacterial community as revealed by network analysis

Christoph A. O. Schmid, Peter Schröder, Martin Armbruster & Michael Schloter

Summary:

In this manuscript, the influence of different agricultural management on the composition and interaction of the bacterial soil community was investigated. In order to be able to reflect long-term effects, an experiment was used that had already been in operation since 1983. In the International Organic Long-Term Nitrogen Fertilisation Trial (IOSDV) in Speyer, two sets of experimental fields were set up with variants of organic soil amendment (harvest residues and horse manure) as well as a control variant without the addition of organic matter. From the additionally available gradient of mineral fertilisation, two were selected for the present study, i. e. 'without' and 'with' mineral fertilisation. In this way, the effects of combined application of organic and mineral measures could be recorded. 16S rRNA gene libraries were prepared from the samples taken and analysed using Illumina sequencing. In contrast to previous studies on the influence of fertilisation or amendment measures, the co-occurrence of bacterial OTUs was investigated in this manuscript. This served as a model for the interactions between the different OTUs. It was first shown that the horse manure treatment had a higher bacterial diversity than the control plots, with bacteria of the taxonomic order Bacillales being typical representatives of this treatment form. In contrast, in the variant with crop residues neither the diversity appeared to be influenced compared to the control, nor could specific organisms for this treatment be identified. From the comparative analysis of the co-occurrence networks of each treatment combination, two additional findings could be drawn. On the one hand, the complexity of the networks was negatively influenced by the addition of mineral fertilisers without simultaneous application of organic amendments. On the other hand, both variants of organic soil amendment, both with and without mineral fertilisation, were able to raise network complexity or weaken the effects of mineral fertilisation. These results show how different types of land use can have a lasting effect on soil microorganisms and underline the importance of sustainable land use for healthy soil life.

Contributions of C.A.O. Schmid:

- contributed to sampling design and sampling
- performed sample preparation, laboratory analyses, sequencing, data analyses
- wrote the manuscript as lead author

3.1.3 Manuscript M3

Impact of high carbon amendments and pre-crops on soil bacterial communities

Catherine W. Kamau, Richard van Duijnen, Christoph A. O. Schmid, Helga E. Balázs, Julien Roy, Matthias Rillig, Peter Schröder, Viviane Radl, Vicky M. Temperton & Michael Schloter

Summary:

The bacterial soil communities of soils amended with high-carbon amendments (HCAs; wheat straw, sawdust, control) were analysed in two different cropping systems (spring barley / winter barley and faba bean / winter barley). The sampling periods chosen were: **T1** after the harvest of the pre-crops (spring barley and faba bean) and **T2** after the harvest of winter barley. The soil bacterial community was analysed from these samples by amplicon sequencing of the 16S rRNA gene. From these data, a notable reduction in diversity was recognised after the final harvest in both the wheat straw as well as the control treatment without HCAs. Sawdust, however, mitigated this effect on diversity. Moreover, soil carbon stocks were increased by sawdust, impacting negatively on crop yields. The bacterial phyla Actinobacteria and Bacteroidetes were favoured by wheat straw and no-HCA control treatments, while Firmicutes, Gemmatimonadetes and Proteobacteria were clearly disfavoured. Generally, the wheat straw and control treatments had similar bacterial communities, differing in only a limited amount ASVs from several phyla. Sawdust changed the abundance of several families of the Proteobacteria compared to the control. Interestingly, the species identity of the pre-crop modified the response of the soil bacterial community only in the wheat straw treament.

Contributions of C.A.O. Schmid:

- contributed to sampling
- supported sample preparation, laboratory analyses, sequencing, data analyses
- critically revised manuscript

3.1.4 Manuscript M4

Potential of wheat straw, spruce sawdust, and lignin as high organic carbon soil amendments to improve agricultural nitrogen retention capacity: an incubation study

Rüdiger Reichel, Jing Wei, Muhammad S. Islam, Christoph A. O. Schmid, Holger Wissel, Peter Schröder, Michael Schloter & Nicolas Brüggemann

Summary:

The nitrogen uptake of winter crops between sowing and the following vegetation period is known to be inefficient. When grown after nitrogen-rich cultivations like canola or faba bean, considerable amounts of nitrogen are lost by nitrate leaching and nitrous oxide emission. High-carbon amendments (HCAs) can prevent nitrogen losses by stimulating carbon-limited microbial growth and thereby biologically fixing the available nitrogen in soil. In this study, an incubation experiment was performed in microcosms containing 8 kg of sandy loam of an agricultural Ap horizon as well as 4.5 t C ha^{-1} in the form of either wheat straw, spruce sawdust or lignin. A control treatment did not receive any carbon addition. To these microcosms, 150 kg NH_4^+-N ha^{-1} were added to generate a high content of nitrogen in the soil. The fastest immobilisation was achieved with wheat straw followed by sawdust. However, this effect weakened in the course of a few weeks. In contrast, no immobilisation of the available nitrogen in the microbial biomass could be observed with lignin. Nitrogen fertilisation as well as the addition of HCAs had an effect on organisms that are significantly involved in the nitrogen cycle, especially ammonium-oxidising archaea and bacteria, but also several denitrifiers. Soils treated with sawdust or wheat straw emitted larger amounts of nitrous oxide or carbon dioxide than control soils. However, this could be balanced against the increased immobilisation of nitrate.

Contributions of C.A.O. Schmid:

- advised in planning and sampling procedures for qPCR study
- performed sample processing, qPCR measurements and data analyses
- critically revised manuscript

4 Discussion

4.1 Land use affects bacterial key taxa and plant-microbe interactions

Every land management measure potentially changes the abiotic conditions in the soil and thus directly affects the living conditions of soil microbial communities. These altered conditions in turn cause changes in the microbial community to some degree. Agriculture offers a wide range of measures that are intended to maintain or improve soil fertility, however, with varying degrees of success (Bai et al. 2018). Understanding the details and context of how agricultural practices affect soil organisms, particularly soil bacteria, could provide a basis for developing improved agricultural systems (Bender et al. 2016). For example, organically managed land showed increased abundance and activity of microorganisms, which could lead to increased sustainability, especially under climate change scenarios (Lori et al. 2017). Precisely because of this enhanced security for the future, it is necessary to develop models for a more ecologically motivated agriculture and to make them economically viable (Geertsema et al. 2016; Geisen et al. 2019b). Understanding the impact of agricultural interventions on soil microbial communities is key to developing sustainable soil management strategies that produce favourable microbial communities for agricultural use.

The restoration sites of the former open-cast mining areas in the Rhineland (manuscript **M1**) are particularly well suited as study objects for investigating short-term impacts. In the course of a few years, several transitions between forms of land use take place there, which are also reflected in the bacterial composition of the soils. Long-term effects of a form of land use can only be meaningfully studied in a permanent field trial. Well-established systems such as the IOSDV in Speyer (manuscript **M2**) with a runtime of several decades are an invaluable scientific resource offering insights into how communities of soil bacteria adapt to a form of land use. Finally, conclusions can be drawn from both types of observations about how soil bacteria interrelate with land use.

With regard to short-term effects, manuscript **M1** showed how a series of changes in land use as part of the restoration measures induced changes of the bacterial soil community. Apparently, the degree of change played a significant role in how abruptly the changes in the bacterial community occurred. Similar results were obtained in other restoration projects, such as the conversion of agriculturally used sites into near-natural ones (Allison et al. 2005; Huang et al. 2011; Barber et al. 2017; Yavitt et al. 2021). Understanding the consequences of changes in entire soil communities is a complex task due to the hundreds of changing organisms. Simplified, but more illustrative and easier to interpret, is the consideration of individual groups of organisms whose role in the overall system is tangible. Using two exemplary cases, I could show the ways in which human influence

might have affected ecologically relevant groups of bacteria. Firstly, there was a higher diversity of OTUs of the order Rhizobiales in early years. This preference of some Rhizobiales indicated the influence of the plants grown on the sites during the first years, which were mainly the legume alfalfa. Legumes have previously been shown to be able to enrich soils with their symbionts (Burghardt et al. 2019; Dinnage et al. 2019). Their well-known role as symbionts in legumes attributes Rhizobiales a central role in agriculture, both for crop plants as well as for commonly used cover crops. Secondly, the occurrence patterns of OTUs from the species-rich genus *Streptomyces* were highlighted. Some of the *Streptomyces* exhibited clear preferences for the different restoration phases and exhibited abrupt changes in abundance within a short time after management transitions. Being closely related species with similar niches, this observation might at least to some extent be explained by the competitive exclusion principle (Kneitel 2008). According to this principle, different strains of *Streptomyces* would be forced to adapt to slightly different niches, as e.g. to different pH optima. Interestingly, a very similar phase preference had been shown for mycorrhizal fungi on the very same sites (Roy et al. 2017).

Regarding long-term effects of different types of management, manuscript **M2** focused on the effects of soil improvement with organic matter. Although the effect of organic additions was found to be lower than that of mineral nitrogen, organisms potentially relevant to the sustainability of the management were also conspicuous in this setting. It had been shown previously, that members of the order Actinomycetales and the class Bacilli were sensitive to changes in fertiliser regimes (Chaudhary et al. 2015; Hartmann et al. 2015; Pershina et al. 2015; Zhu et al. 2016). Adding to this, bacteria of the genus *Bacillus* had been found before to be indicative of manure-amended farmland (Chu et al. 2007). Consequently, members of the order Bacillales, namely the families Planococcaceae, Thermoactinomycetaceae and Turicibacteraceae, were identified in manuscript **M2** as the main responders to manure applications, as well as Gemmatimonadetes and Clostridiales to a lesser extent. In the meantime, this result has been confirmed in similar settings, again highlighting Bacillales, but also Clostridiales, as typical followers of manure application (Lin et al. 2019; Ye et al. 2019). Since all those results have been obtained in different soil types and climate zones, these findings seem to be robust patterns of long-term manure application worldwide.

In addition to the taxonomic identities of the organisms, manuscript **M2** also provided insights into the coexistence of bacteria. This was done on the model of network analysis. An increased complexity of the bacterial networks in soils amended with organic materials when compared to soils receiving no organic inputs was demonstrated. Also this result has recently been confirmed in a long-term trial with an acidic Ultisol in China showing increased bacterial co-occurrence network complexity in soils receiving pig manure as organic input (Ye et al. 2021). Besides manure, plant

residues raised network complexity in manuscript **M2** to a similar extent despite no visible effects on the α-diversity nor the community composition.

At this point, the question arises to what extent these observations could be relevant for the sustainability of management. A critic might argue that soil microbial communities have a high degree of functional redundancy and that changes in soil communities would therefore be neutral to the outside world (Louca et al. 2018). This redundancy may indeed be the case for many soil functions. However, in crop production, interactions between microbes and plants often play an essential role – both positive and negative – in plant health and thus crop yields and profitability (Oleńska et al. 2020; Vishwakarma et al. 2020). Depending on the degree of specialisation in such interactions, the growth-promoting effect on a given crop might constitute a phylogenetically narrow soil function (Schimel & Schaeffer 2012). As such, it could be lost in the course of land-use changes. In addition, there appear to be key taxa that play an extraordinary role in crop yield (Gu et al. 2020). Irrespective of yields and crop performance, there are key taxa in the soil bacterial community that strongly influence the composition and function of the entire population (Banerjee et al. 2018). In the manuscripts of this thesis several groups of organisms have been identified that responded to anthropogenic impacts. Those were Rhizobiales and *Streptomyces* in manuscript **M1**, Bacilli, Clostridiales and Gemmatimonadales in manuscript **M2** as well as *Bacillus*, *Gemmatimonas* and *Streptomyces* in manuscript **M3**. It is noticeable that these include key taxa of the soil microbial community as well as known plant growth-promoting rhizobacteria (PGPRs) (Banerjee et al. 2016; Banerjee et al. 2018; Gu et al. 2020).

The role of Rhizobiales as nitrogen-fixing symbionts of legumes is a well-established example of plant-microbe interactions (Cararetо Alves et al. 2014; Sugiyama et al. 2015; Sugiyama et al. 2017). Both the Rhizobiales and the streptomycete community in soil were shown to be selectively shaped by plants (Bakker et al. 2013; Jauri et al. 2018; Burghardt et al. 2019; Dinnage et al. 2019; Hannula et al. 2021). Also for *Streptomyces*, close interactions with plants were suggested in literature, since they can be found as endophytes in roots both in model systems and in crop plants under field conditions (Bulgarelli et al. 2012; Edwards et al. 2015; Worsley et al. 2020). In transplant experiments, *Streptomyces* exhibited strong plant growth-promoting effects (Gu et al. 2020). While as endophytes they might act as PGPRs, streptomycetes were also discussed to act as biocontrol agents against plant pathogens due to the rich spectrum of secondary metabolites the genus produces (Viaene et al. 2016; Abbasi et al. 2021). Lastly, *Streptomyces* are known to decompose recalcitrant organic materials, e.g. lignocellulose or chitin (Crawford 1978; Williams & Robinson 1981; Riyadi et al. 2020; Tippelt et al. 2020). As such they play a role in the decomposing community and contribute to the carbon cycling in soil ecosystems.

As mentioned above, Bacillales and Clostridiales are typical followers of manure application as was the case in manuscript **M2**. The preference of these orders for manure may be explained by

the increased amount of macroaggregates in the soil when organic soil amendments are applied (Annabi et al. 2011). The aggregates increase spatial heterogeneity in the soil and create anaerobic habitats for the Clostridiales (Hansel et al. 2008; Zhang et al. 2015). But the Bacillales also seem to have an affinity for macroaggregates (Fan et al. 2021a). Since some Bacillales produce extracellular enzymes, e.g. cellulales, polyphenol oxidases or lipases (Gupta et al. 2004; Mayende et al. 2006), they might play a role in the turnover of soil organic matter (Sinsabaugh 2010; Duan et al. 2021). In fact, the genus *Bacillus* has been hypothesised before to accelerate carbon and phosphorus cycling in soils and in this way play its role in the observed increased yields in manure-amended farmland (Feng et al. 2015). The genera *Bacillus*, *Paenibacillus*, *Planococcus* and *Thermoactinomyces* have further been identified as possible PGPRs via various mechanisms (Govindasamy et al. 2011; Rajput et al. 2013; Verma et al. 2016; Ajar et al. 2017; Sansinenea 2019; Tiwari et al. 2019). In this way, amending farmland with manure might not only introduce nutrients for the crops, but also arrange favourable soil microbial conditions. Thus, organic amendments can have a double benefit from the crop's point of view. In contrast to the previously mentioned taxa, our knowledge about the role of Gemmatimonadetes in soil is very limited. We only know that they prefer dry, aerobic conditions and that cultivable representatives act as denitrifiers, especially in the reduction of nitrous oxide to elemental nitrogen (Park et al. 2017; Chee-Sanford et al. 2019).

4.2 Soil bacteria and restoration measures

Land-use changes are not always transitions between agricultural forms of use. Reclamation and renaturation measures can also be regarded as changes in land use or as anthropogenic influence and they will thus change the microbial soil community as a result (van der Heyde et al. 2020). The extreme case of the restoration of former opencast mining areas illustrates the extent to which soil bacteria can be affected by human intervention to soil ecosystems. Various examples from the literature demonstrate, that restoring soil to its original state after open-cast mining is an unrealistic goal (Insam & Domsch 1988; Dunger et al. 2004; McKinley et al. 2005; Jangid et al. 2010; Lewis et al. 2012; de Quadros et al. 2016; Ezeokoli et al. 2020; Lane et al. 2020). Instead, the actions of mining and subsequent restoration leave their imprints in both the abiotic soil characteristics and the microbial community for extended periods of time. Examples of other types of reclamation projects, such as the restoration of wetlands on formerly agricultural land (Yavitt et al. 2021), the restoration of sand dune systems (Yu et al. 2020) or coastal areas (Yan et al. 2020), describe that reclaimed sites may still differ from natural sites in their microbial composition even after many years. Also on the areas of manuscript **M1**, the goal of making the land usable again is achieved. However, a strongly altered soil community remains.

The goal for the agricultural restoration in the Rhineland area was primarily defined in terms of the usability, emphasising the role of agricultural land in the region. For example, on the website of RWE Power AG it states that the regional unmined soils are “among the most fertile and best sites in the world.” While acknowledging its “high level of responsibility for the production process of [...] new land soils”, the operator merely identified the “proper use of the loess produced at the surface” as the primary means “to achieving a high yield” (all quotations cf. OR 6: RWE Power AG, 2021). This focus on yield, however, ignored the fact that the external conditions for soil life in the newly created areas were fundamentally different from the original agricultural areas. In this way, the form of use after restoration was externally comparable to before, but the soil conditions caused significant and persistent changes in the bacterial soil community compared to the original soils (manuscript **M1**).

Microorganisms, their diversity and their activity are of central importance for the metabolic cycles in the soil and thus literally prepare the basis for successful agricultural use (Kibblewhite et al. 2007; Hirsch & Mauchline 2015; Sokolov et al. 2020; Barnett et al. 2021). A loss in microbial diversity can affect soil functions and jeopardise the ecosystem’s stability (Wagg et al. 2021). Still, the properties of the restored soil differ significantly from the original, unmined soil (cf. table 1). In fact, simply because of the history of their formation, these soils could be described as an anthropogenic artefact that would not arise in this form from natural processes (Capra et al. 2015). This notion raises questions about the sustainability of restoration. One such aspect is the resilience or resistance of soil communities to temperature or precipitation extremes, which are expected to increase in temperate latitudes due to climate change (National Academies of Sciences, Engineering, and Medicine 2016; Stott 2016). So far, we have not fully understood how soil microbes respond to such extremes (Bardgett & Caruso 2020). A change in the prevailing soil community due to human intervention in the context of restoration and renaturation could bring with it additional imponderables that are still completely unknown. Whether a new land soil on a former open-cast mine site is as resistant and resilient as an old land soil, e.g. to drought, remains to be seen in future studies. With regard to restoration, the microbial properties of soils should definitely be included in the target setting as well as the follow-up investigation, e.g. by complementing non-molecular measurements with genomics data (Garris et al. 2016; Hart et al. 2020).

Table 1: *Soil characteristics of original and restored soil in the Rhineland mining area.*

Data were partially reproduced from OR 7. Top soil (0 – 10 cm depth) values for lime and humus content are measurements from manuscript ***M1****.*

property	original soil	restored soil
age	~ 13,000 years	1 – 70 years
top soil	medium to strongly clayey silt	medium clayey silt
subsoil	strongly clayey silt	medium clayey silt
pH	~ 6.5	~ 7.5
lime content	21 % (top soil 0.1 %)	~ 7 – 21 % (top soil 0.7 – 11.0 %)
humus content	1.6 – 1.8 % (top soil 2.2 – 2.5 %)	0.4 % (top soil 0 – 1.9 %)
usable field capacity	200 – 220 l / m²	220 – 240 l / m²
nutrient content	medium to high	low to medium
microbiological activity	high	low
yield potential	high to very high	high to very high

4.3 Organic amendments as ecosystem engineering tools

Due to their ability to influence soil properties in the long term, organic amendments are suitable tools for optimising agricultural systems. Organic amendments can strongly influence key parameters of anthropogenic ecosystems such as biodiversity and the complexity of microbial networks (Ye et al. 2019; Ye et al. 2021). They can thus be considered a tool for engineering the diversity and functionality of ecosystems to improve the sustainability of their use. Examples for organic materials used in improving soil are manure, biosolids and high-carbon amendments such as sawdust or biochar. In manuscript **M2**, horse manure and crop residues were shown to improve the soil bacterial community. Very similar effects were obtained with biosolids (Schlatter et al. 2019). The effects of these amendments included an increased occurrence of the taxa *Bacillus*, *Turicibacter*, Planococcaceae (cf. manuscript **M2**) and *Streptomyces* (cf. **M1**). Due to the similarity of biosolids compared to manure, this result was to be expected. Other organic materials can also influence the taxa mentioned. For example, the results of manuscript **M3** demonstrated the positive effects of sawdust on the abundance of some Bacillales and Gemmatimonadetes, while Streptomycetaceae were affected negatively. What is more, evidence in the literature on biochar suggests its use on farmland to affect a similar set of taxa, namely Bacillales, Clostridiales and the Streptomycetaceae among others (Cole et al. 2019). Taking both manuscripts **M1** and **M2** as well as the evidence from literature into account, the bacterial groups of Bacillales, the streptomycetes and to a lesser extent Clostridiales and Gemmatimonadetes might represent bacteria with a high sensitivity towards different agricultural use forms. As such, they might in the future prove to be suitable candidates for a microbial monitoring of farmland to assess the current status of soil health. In addition to that, comparable results from forest ecosystems suggest generalisations beyond intensively used farmland (Xue et al. 2020). Interestingly, biosolids have recently been

shown to enhance defence responses in plants and disease suppression in soil (Stavridou et al. 2021). This plant-protective effect further illustrates the positive influence of organic amendments on the sustainability of land use, since they might be used to generate favourable microbial conditions for plant cultivation. In particular, the effects of organic amendments on Bacillales and streptomycetes are of special interest due to the role of these organisms in promoting plant growth (as described in 4.1).

The use of organic amendments has further been shown to have effects on the soil functional level. In manuscript **M4**, we demonstrated effects of different high carbon amendments on nitrifiers and de-nitrifiers as well as greenhouse gas emissions and nitrogen immobilization in soil highlighting the different properties of wheat straw and sawdust for these purposes. However, ammonia-oxidising archaea and bacteria can exhibit different reactions and sensitivity towards organic amendments, probably depending on the type of amendment applied as well as the soil type (Yang et al. 2020b; Tao et al. 2021). While it is often proclaimed to counteract nitrate leaching by applications of high carbon amendments, the resulting management options need to carefully balance with other factors like irrigation and fertilisation to prevent increased N_2O emissions (Zhao et al. 2021). Beyond that, nitrogen fixation activity has been shown to increase by compost applications (Chen et al. 2020). Remarkably, the bacterial diazotrophic subcommunity studied by the aforementioned authors showed similar trends in terms of increased network complexity due to organic inputs as the entire bacterial community in manuscript **M2**, with *Rhizobium* being a hub species in the network of diazotrophs. These results provide the basis for optimisations to land use forms, generating a catalogue of measures to adjust soil functions to desirable levels while making use of the underlying microbial activity.

4.4 Human impact on fungi, protists and nematodes

While this thesis was concerned with the bacterial subset of the soil microbial community, literature provides ample evidence for impacts of different soil amendment strategies on the entire soil life. Archaea, at least the ammonia-oxidising, were shown to be influenced more strongly by additions of organic amendments than bacterial ammonia oxidisers (Tang et al. 2021), likely due to the effect of manure on soil pH. On the same sites as those used in manuscript **M1**, effects of agricultural land use have been shown to impact on the community structure of arbuscular mycorrhizal fungi (Roy et al. 2017). Interestingly, manure additions seem to have similar effects on the fungal community as discussed above for bacteria, mitigating adverse effects of mineral fertilisation (Ding et al. 2017). Woody materials are suitable to increase the saprophytic fraction of the fungal community (Clocchiatti et al. 2019). When used as soil amendments, organic materials and biochar have been repeatedly found to reduce the abundance of plant pathogenic fungi in soil (Ding et al. 2017; Yao et al. 2017; Tayyab et al. 2019). As a parallel to manuscript **M2**, organic materials were

recently shown to enhance the complexity of fungal co-occurrence networks (Ji et al. 2020). Also for protists a positive impact of organic soil amendments on their diversity has been identified (Guo et al. 2018). As shown for bacteria and fungi, the protist community can be altered to contain less plant-pathogenic organisms using soil amendments (Xiong et al. 2018). Lastly, nematodes can be influenced by organic materials with effects depending on application rates (Pan et al. 2020). Taken together these results clearly underline the effect of soil improvement measures with inputs of organic materials on the entire soil life involving all important groups of organisms. For agricultural systems this offers possibilities to generate crop-friendly microbial soil conditions, e.g. by reducing the amount of plant pathogenic organisms in favour of plant growth-promoting ones. In restoration contexts these findings might contribute to holistic approaches starting projects with the preparation of suitable soil biotic conditions for the to-be restored ecosystem.

4.5 Further factors of human influence

The factors of human influence on soil bacterial / microbial communities are very diverse and only a small portion of them has been studied in this thesis. Comprehensive studies investigating the combined effects of multiple factors are rare due to experimental difficulties. The results of manuscript **M1** underline this point since even the similar use forms of RWE Power AG and the local farmers led to a considerable change in the bacterial soil communities. A promising approach seems to be a classification of the diverse factors of human influence into similar groups to narrow down the amount of factors to be studied in future experiments (Rillig et al. 2021). Besides fertilisation and soil amendment, mechanical tillage is one of the best-studied factors of human influence on soil life. Even single applications of the plough were able to influence the microbial composition of the soil (Kraut-Cohen et al. 2020). With permanent application, changes in soil chemical properties were coming into play. Ploughing affected both the structure of the soil microbial community and its enzymatic activity (Gabbarini et al. 2021). Interestingly, organisms that reacted positively to the absence of ploughing tended to be found in the coarser soil particles and, conversely, organisms that reacted positively to regular ploughing tended to be found in the finer soil particles. In addition, they were each involved in different material cycles. This effect on the different particle fractions might at least partly be explained by different abundances of lipopolysaccharide-producing organisms in ploughed and unploughed soils (Cania et al. 2020). Such findings are also relevant from an economic perspective. Ploughing partially breaks up the depth profile of soil, which also impairs soil functions important for plant growth, such as nitrogen fixation. This is because nitrogen-fixing organisms differed across the depth profile (Wang et al. 2021). By ploughing, this pattern is disrupted. The effects of this apply equally to bulk soil and the rhizosphere (Hu et al. 2020; Li et al. 2021), whereby reduced ploughing had a positive effect on diversity and co-occurrence patterns for nitrogen fixers. In order to optimise land management, it is important to recognise as many of these influencing variables as possible and thus to weigh up the

different aspects against each other. For example, while one result of manuscript **M2** was that bacterial soil diversity and the abundance of organisms potentially beneficial for plant growth increased with the addition of manure, manure simultaneously inhibited the growth of diazotrophic organisms (Lin et al. 2018). The bottom line is that manure application could create a microbial environment with lower nitrogen fixation potential in the long term. This lowered potential, in turn, can lead to fertiliser requirements for crops increasing over time, leading to conflicts that need to be weighed against each other. While other factors are much less well studied in terms of their effects and modes of action, similar trade-offs can be expected from other combinations of management practices. As an example, it is known that irrigation can affect bacterial communities (Bastida et al. 2017). But also the use of pesticides is already known to alter microbial community composition (Bragança et al. 2019). Here, too, there are some indications that a combination with organic improvement measures modulates the effects of pesticide application (Álvarez-Martín et al. 2016; Pose-Juan et al. 2017). These examples reveal the complexity of the topic and show that statements on isolated measures in land management are likely to have only limited significance in practice. Further studies that take into account the entire range of human influence are therefore indispensable for optimising our use strategies in terms of environmental protection and sustainability. As a starting point, manuscript **M1** demonstrated how a change in land management, through either a change in land use type or the specifics within a land use type, affected the microbial community and identified responding taxa to both long-term site development and specific use characteristics. Building on this work, the main drivers of these changing can be further narrowed down to reveal the mechanisms behind these observations.

4.6 Microbial interactions as influenced by human action

While the effects of human actions on soil microbes are frequently studied at a high level, knowledge of how external factors affect biotic interactions between organisms, e.g. predator-prey or symbiotic relationships, is lacking. There are now many observations on how microbial soil communities react to human influences, i.e. change in their diversity, composition or the topology of their co-occurrence networks. These findings are important and relevant for the development of novel management options. However, an important building block for the actual understanding of the processes in soil microbial communities is missing, namely the knowledge of the ways in which these organisms interact with each other and the ways in which these interactions are influenced from the outside. These external impacts could be direct human interventions, as e.g. by land management practices as discussed above, or indirect effects, e.g. by interactions between soil organisms and (crop) plants (Thies et al. 1995; Bakker et al. 2013). Although it has been shown several times, as described above, that network topologies can change as a result of such external factors, there is no direct evidence of what the exact causes as well as consequences are for the soil system as a whole.

With the formation of microbial guilds, we know examples of how positive interactions can look in detail even spanning kingdoms (Raghoebarsing et al. 2006; Jones et al. 2014). For example, the degradation of plant material gives rise to microbial groups that interact like units in the degradation process (Moorhead & Sinsabaugh 2006). These guilds may differ according to the chemical characteristics of the decaying material. Importantly, the formation of such guilds can be modulated by additions of fertiliser and in turn influence decomposition processes (Bhatnagar et al. 2018). On the side of negative interactions, competition for limited resources is one possible mechanism and examples like the production of antifungal volatiles by bacteria provide a glimpse of how complex interactions can be in the species-rich soil environment (Li et al. 2019c). However, knowledge about the dynamics, mechanisms or conditions of these interactions is often incomplete. As a show case, predator-prey relations ships are well known from microbiology (Varon & Zeigler 1978; Casida 1988; Wang et al. 2011; Duncan et al. 2018). *Ensifer adhaerens*, for example, is a facultative bacterial predator. Yet, its capabilities of lysing host cells is pH dependent (Casida 1982; Jurkevitch & Davidov 2007). Similarly, *Cupriavidus necator* attacks its bacterial prey, including ecologically relevant taxa such as *Bacillus* or *Streptomyces*, only when the availability of nutrients in soil decreases (Makkar & Casida 1987; Jurkevitch & Davidov 2007). With fertilisation and organic amendment of agricultural soils acting on both soil pH and nutrient levels, these might be small pieces of the mosaic to understand what is happening on the organism level when we observe co-occurrence networks to differ between management types. With land management practices – including organic inputs and tilling – not only impacting on the bulk soil, but also on functional microbial groups in the rhizosphere communities and, ultimately, crop yields (Liu et al. 2019; Bziuk et al. 2021), the relevance of this knowledge for sustainability quickly becomes clear.

4.7 Evaluation of the hypotheses

Hypothesis **H1** states that changes in the soil bacterial community caused by changes in land use are (1) of significant magnitude, (2) have a long-term effect and (3) could have an impact on the sustainability of use. Regarding (1), it could indeed be shown in manuscript **M1** that a similar degree of change in the soil bacterial community is achieved through changes in use as happens through seasonal variation. Long-term effects (2) could be detected in manuscript **M2** in particular. In the permanent field experiment, soil communities with different characteristics established themselves over long periods of time, depending on the characteristics of management. With regard to the sustainability of management (3), there is evidence in manuscripts **M1**, **M2** and **M3** of changes in key taxa as well as organisms that interact closely with plants. While this does not directly question sustainability, these findings at least provide scope for future research questions.

Human influence on the bacterial soil community is also evident against the background of ecological restoration. Hypothesis **H1** seems to be viable from this point of view as well. In many

restoration projects – also on the areas of manuscript **M1** – changes in the soil community caused by land use remain visible after restoration measures. In analyses, natural and restored sites can be distinguished on the basis of their microbial composition. This condition often lasts for decades, and restored areas return to their natural state only slowly, if at all. Lastly, there are unanswered questions with regard to sustainability, especially on former opencast mining areas, which future research will have to answer. For this, the interrelationships between the microbial soil community, external environmental influences and the vegetation must first be better understood in the overall picture.

Hypothesis **H2** states that with a suitable selection of agricultural methods, the bacterial soil community can be changed in favour of increased sustainability of the system. For this statement, manuscripts **M2**, **M3** and **M4** provide examples of how soil life can be specifically influenced with the addition of various organic substances. Some of these measures, such as the addition of manure, are common practice. However, understanding how these measures work gives us the opportunity to learn about the factors that make up sustainable soil management. In the future, we should try to identify other tools that can be used to influence soil life in a targeted way. In this way, new forms of management can be devised that are better able to implement the concerns of sustainability.

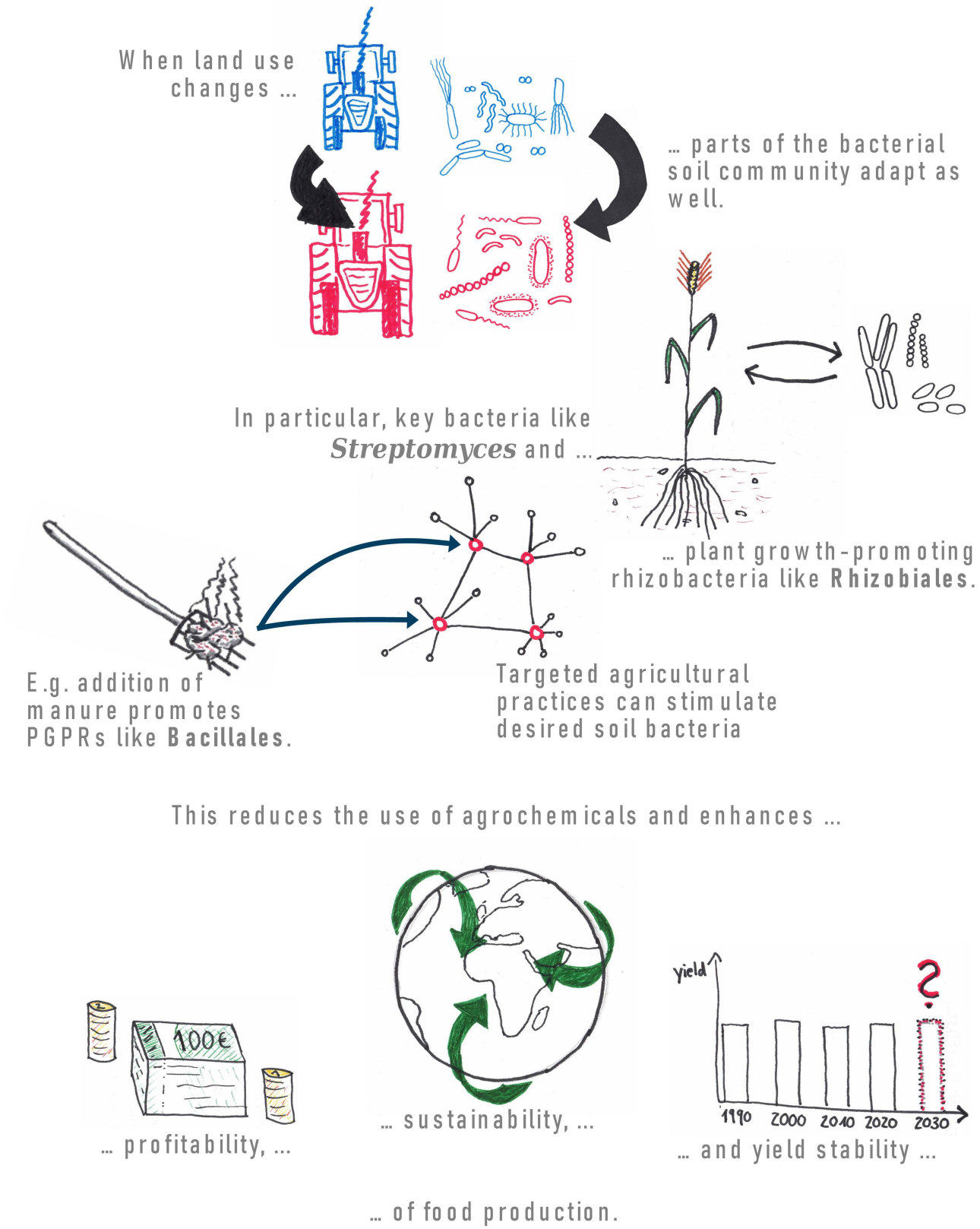

Figure 4: *Key messages as a sketchnote*

This sketch summarises the main findings of this thesis: (I) when land use changes, the soil bacterial community will adapt to the new conditions. (II) Most importantly, key taxa of the community change with land use with so far unknown consequences for land management. (III) Agricultural management practices can be used to adjust the soil bacterial community. These findings may ultimately help to increase the sustainability of agricultural production.

4.8 Conclusions and Outlook

Despite the millennia-old history of agriculture, reconciling economic efficiency with conservation of natural resources is a more pressing issue than ever. Mass extinctions due to the consequences of the Anthropocene, caused in part by agriculture, remind us of the need for sustainable food production (Cowie et al. 2022). While in recent decades there has been a growing awareness that healthy soils are a limited resource that require prudent management, there is still a lack of knowledge for effective adaptation of soil management. Many general aspects concerning the material cycles in the soil have been studied and understood in the meantime, including the important role of microorganisms for soil function and fertility. Looking at our crops in isolation without considering their interactions with harmful as well as growth-promoting soil microbes makes little sense. Moreover, since more detailed analyses of soil microbial communities have become possible, it has also become increasingly clear that the composition of these communities and the interaction between the microorganisms themselves are of great importance for vegetation or crop production.

This doctoral thesis took up this point and, based on the group of bacteria, addressed the question of the role humans play in this system and the ways in which their influence manifests itself in this complex system. For agriculturally used sites, it could be shown that this influence is of significant magnitude and that the soil bacterial communities quickly adapt to changed conditions in cultivation (Figure 4). After the initial changes in use, the bacterial communities remained stable within the limits of seasonal fluctuations under constant management. What is remarkable, however, is the observation in the various studies that, due to changes in agricultural practices, those bacteria change that could play a special role for the cultivation of crops. Among them are plant growth-promoting organisms such as Bacillales or *Streptomyces*, but also well-known symbionts such as Rhizobiales. These results show the ways in which the type of cultivation could have an impact on the economic success, but also on the sustainability of the use. However, more research is needed on this before the consequences of such changes can be meaningfully assessed. Especially with regard to plant-microbe interactions, the extent to which these are affected, for example, after a change in soil management, has not yet been adequately researched. Research on fresh-water microbial systems suggests that such specialised functions might not be redundant and might be lost from the system as a result of local extinction (Delgado-Baquerizo et al. 2016). Likewise, the dynamics of the material cycles could possibly be influenced in a way that is unfavourable for the intended use and thus reduce the sustainability of the management. In fact, recent evidence suggests key-stone taxa to represent important steps in soil nutrient cycling, including carbon, nitrogen, phosphorus and sulphur cycles, and by that being directly connected to actual crop yields (Ling et al. 2016; Fan et al. 2021b). Investigating the effects

of bacterial community changes for our agricultural systems and the potential exploitation of these changes will therefore be a challenge for future scientific work in microecology.

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List of abbreviations

ANOVA	analysis of variance
ASV	amplicon sequence variant
bp	base pair
CAP	constrained analysis of principal coordinates
DM	dry matter
dsDNA	double stranded deoxyribonucleic acid
ESS	effective sample size
FM	fresh matter
FP	forward primer
GPS	global positioning system
HCA	high-carbon amendment
HDI	highest density interval
IOSDV	internationaler organischer Stickstoffdauerdüngungsversuch (international organic nitrogen fertilisation trial)
LMM	linear mixed model
LUFA	Landwirtschaftliche Untersuchungs- und Forschungsanstalt
MCMC	Markov chain Monte Carlo (methods)
NCBI	National Center for Biotechnology Information
NPK	nitrogen phosphorus potassium (fertiliser)
OTU	operational taxonomic unit
PGPR	plant growth-promoting rhizobacteria
(q)PCR	(quantitative real-time) polymerase chain reaction
qq (plot)	quantile-quantile plot
rDNA	ribosomal deoxyribonucleic acid
RDP	Ribosomal Database Project
RP	reverse primer

rRNA	ribosomal ribonucleic acid
SD	standard deviation
WHC	water holding capacity

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Appendix I: Manuscript M1

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52 years of ecological restoration following a major disturbance by opencast lignite mining does not reassemble microbiome structures of the original arable soils

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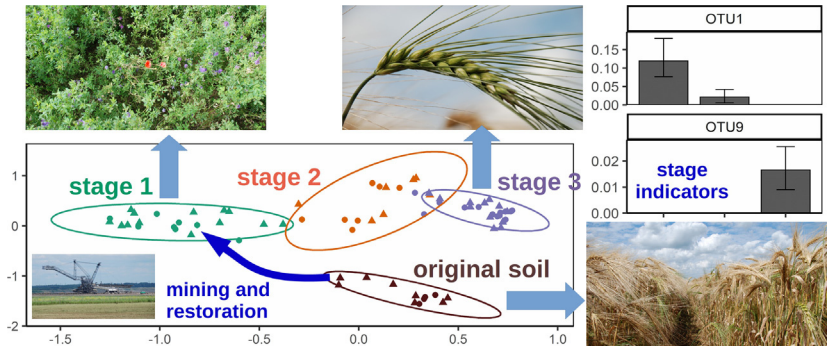
HIGHLIGHTS

- Land management during ecological restoration has a major impact on soil microbiome.
- Preference of organisms for specific management type might influence soil functions.
- Meta-analysis reveals trends during restoration independent of management procedures.

GRAPHICAL ABSTRACT



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ABSTRACT

Opencast mining for lignite continuously creates areas of land that require restoration. Here we applied a chronosequence approach to investigate the development of soil bacterial communities during 52 years as influenced by the restoration process and subsequent changes in soil physico-chemical conditions starting from the initial reclamation of the sites. By comparison with the unaffected soils near the mine, we were able to address the question if soil bacterial communities have reached a steady state within 52 years, which is comparable to the original soil. Our study revealed three distinct phases of the restoration process, each with a specific bacterial community composition. The effect size of these changes was similar to the one observed for seasonal dynamics at our sites. At the beginning of the restoration process Flavobacteriaceae, Cytophagaceae and Sphingobacteriaceae were found as typical members of the bacterial community as well as Rhizobiales as a result of the cultivation of alfalfa on the restored plots. At later stage the families Peptostreptococcaceae, Desulfurellaceae as well as Streptomycetaceae increased in relative abundance and became dominant members of the bacterial community. Even though overall bacterial abundance and richness exhibited values comparable to the original soil already 5 years after the start of the restoration process, main responder analyses reveal differences in the bacterial community structure even 52 years after the start of the restoration process. Mostly Nitrospirae were reduced in abundance in the soils restored for 52 years compared to the original soils. To broaden the significance of our study, we compared our data bioinformatically with published results from other restored areas, which were

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previously affected by opencast mining. Despite different durations of the different restoration phase, we could observe a large degree of conformity when bacterial patterns of succession were compared indicating common modes of action of ecological restoration tools for bacterial communities.

1. Introduction

As a consequence of human activities, the Anthropocene is characterised by altered ecosystems with severe consequences for biodiversity and ecosystem services (Temperton et al., 2019). Ecological restoration is gaining traction as complementary tool for biodiversity conservation, which allows for the re-establishment of ecosystem functions and services, mainly in areas with highly disturbed or destroyed ecosystems (Bullock et al., 2011). The underlying concepts are well established for macroecology but information on consequences of restoration approaches for microecology is still rare.

Only recently, some basic ideas about the succession of microorganisms in soils during natural restoration have been described. Especially phototrophic organisms from the phyla Chlorobi, Chloroflexi and Cyanobacteria as well as nitrogen-fixating bacteria characterise early succession stages (Urbanová et al., 2011; Deonalli et al., 2017). In addition, organisms from the phyla Proteobacteria and Firmicutes were described as early colonisers of disturbed ecosystems. The subsequent development of bacterial communities from the initial stage is partly of stochastic and partly of deterministic nature (Dini-Andreote et al., 2015). Regional climatic conditions as well as the local history of the substrate impact on these trends (Jangid et al., 2011; Liang et al., 2015). In addition, changing soil chemistry plays an important role in shaping bacterial communities. Mainly the soil pH acts as a strong driver for bacterial community composition, as even within the same taxon different pH optima have been described (Kielak et al., 2016; Lewin et al., 2016; Bhatti et al., 2017). Accumulating organic carbon in soils and its mutual influence on microbial communities is another driver for succession (Naveed et al., 2016; Don et al., 2017). The so far published data mainly refers to the initial states of restoration or describes natural succession patterns, without human intervention (Schulz et al., 2013). The impact of human intervention on soil communities during a complete restoration cycle, which often spans many decades, has hardly been studied. However, an in-depth understanding of the overall responses of the soil microbiome towards restoration approaches during a complete restoration cycle might help to generate positive feedback loops and trigger multifunctionality of the affected landscapes and the associated ecosystem services.

Chronosequence-based approaches enable the investigation of the development of ecosystems on a decadal to millennial scale, covering initial stages as well as the resulting climax systems (Walker et al., 2010). Opencast mines have been established all over the world for the extraction of coal or minerals from near-surface deposits. As mining and restoration are commonly happening concurrently, opencast mines represent ideal study areas for man-made chronosequences. As such, they allow the analysis of sites from different stages of restoration within a defined area, together with the original status of the ecosystems before being affected by mining.

In this study we used a chronosequence of sites from a former opencast lignite mine, to get a deeper understanding about the role of ecological restoration for the succession of microbial communities. The sites had been subjected to a progressive ecological restoration with the aim to re-generate land for agriculture – the typical form of land use in this area. With our sampling strategy we spanned a time period of 52 years from the initial substrate used for restoration towards land under agricultural use. Our approach also included reference sites which were under agricultural use but not affected by mining. We focussed on bacterial communities, which were characterised using molecular barcoding. We assessed the size of the land management effects using seasonal dynamics as a baseline. We further compared our data to three other published studies, which used different forms of ecological restoration, to assess the impact of the type of man-made restoration on bacterial succession patterns. Finally, we defined tipping points in microbial community dynamics, which are independent from the concrete form of man-made restoration procedures.

2. Materials and methods

2.1. Site description

For this study a restoration area of a former opencast lignite mine operated by the RWE Power AG (https://www.group.rwe/) near Inden, south of Jülich, Germany (50° 52′ 46″ N, 6° 19′ 21″ E) was chosen. The mean annual temperature is 9.8 °C and the total annual precipitation is 829 mm according to the Deutscher Wetterdienst (https://www.dwd.de). For mining, the original soil and the underlying overburden materials were removed by stratified digging, covering a depth profile of roughly 6 m. For restoration, the loess material obtained during the digging process was mixed with top soil from the same site, which has been characterised as a Luvisol (ratio 5:1) and immediately transported via conveyor belts to the dumping site at the back of the mine. Both mining and restoration are happening concurrently and continuously, using the soil material of the digging front without delay as substrate for restoration. Because of the continuous mining and restoration since 1957, a chronosequence of sites with different developmental stages are available in a tight area, which are based on comparable parental materials. Overall, the restoration process is divided into three phases: during restoration stage 1 (RS1), after a three months recovery phase, which resulted in the formation of an initial soil profile of about 2 m depth, the dumped soil material is cultivated continuously with alfalfa (*Medicago sativa*) for three years. Since the soil substrate at this stage is characterised by low organic matter content, and lacks soil structure, the soil fertility of these materials is low, which makes an initial cultivation phase necessary to prepare the sites for later agricultural use. Alfalfa has been suggested by RWE at early vegetation stages to (a) restructure the soil, (b) to enrich it with organic residues, and (c) to re-initiate biological activity. After this initial phase, the sites are suitable to support the cultivation of cereal crops. Besides mulching with alfalfa and a final compost application, no mineral fertilisation or biocide treatments are performed. In autumn of the third year, alfalfa fields are tilled, which marks the end of RS1. During restoration stage 2 (RS2), the operating company, RWE Power AG, starts to cultivate winter wheat (*Triticum aestivum*), followed by winter barley (*Hordeum vulgare*), canola (*Brassica napus*), and sugar beet (*Beta vulgaris*). Mineral fertilisation rates per hectare and year usually range between 180–210 kg N, 45–100 kg P, 45–130 kg K, and 0–20 kg Mg. Restoration stage 3 (RS3) starts with the return of the sites after seven years restoration activity to local farmers, who usually continue the crop rotation with focus on sugar beet cultivation, following region-typical agricultural fertilisation and plant-protection guidelines.

2.2. Sample selection and sampling procedure

We performed two samplings in March 2016 and June 2016 at the same GPS positions (Fig. S1). In total, nine sites along a 52-year restoration chronosequence, comprising all restoration stages described before were sampled: the freshly deposited substrate from 2016 (sampled once in June), RS1 cultivated with alfalfa, restored in 2015 (sampled

once in June), 2014, and 2013; RS2 cultivated with barley, restored in 2012, still managed by RWE Power AG; and RS3 managed by local farmers, restored in 2006, 1990, 1979 and 1964 and cultivated with winter wheat. For comparison, we also included a reference site close to the digging front, representing the original soil status prior to mining (OS). Since the reference site was in immediate proximity to the front of the mining pit, its characteristics well represented the soil material used for creating the substrate and restoration of the youngest restoration sites of the chronosequence. Furthermore, the reference sites were cultivated with winter wheat, ensuring a comparable vegetation with plots from RS2 and RS3. The selected field sites had an average area of six ha each. We collected four composite soil samples from the upper soil horizon (0–10 cm) from each site at an average distance of 115 ± 32 m (mean ± SD). Each composite sample consisted of five sub-samples of 500 cm^3 taken within a 5 × 5 m square around the GPS-marked position. Composite samples were vigorously mixed and sieved, approximately 300 g were stored at 4 °C for physico-chemical analyses, while another 10 g were immediately sieved through a 2 mm mesh with sterilised tools and stored on dry ice prior to freezing at −80 °C for microbial analyses.

Further details on abiotic soil properties of the different sites are listed in Table S1 and have been described also in Roy et al. (2017). All soil samples had a comparable silty loam soil texture with 17% clay, 78% silt, and 5% sand in average. The bulk density was between 1.2 (freshly deposited) and 1.4 g m^{-3} (original Luvisol soil). The pH_{CaCl2} of the original soil was slightly acidic and depleted of $CaCO_3$. The restored soils initially contained considerable amounts of $CaCO_3$ buffering the pH at alkaline levels. Ten years after restoration, $CaCO_3$ contents decreased and more neutral soil pH values were measured. Organic carbon increased with time after restoration, particularly after transfer from alfalfa cultivation to conventional agricultural practice (RS2 and 3). In soils from RS3, C_{org} was clearly enriched. Also, total N clearly accumulated with time of restoration. This effect was particularly strong in RS2. However, both C_{org} and total N remained below the values found in the original soil. Contents of ammonium and nitrate peaked in the March sampling of soils restored in 2006 and 1990, as well as in the original soil, whereas in the remaining sites only low levels were found. Plant accessible P increased stepwise in soils from RS2 and RS3 to levels comparable to the original soil. Likewise, total P content increased with time of restoration, particularly in RS3, but remained below total P of original soil.

2.3. DNA extraction and quantification of 16S rRNA genes

Total DNA was extracted from 0.25 g fresh weight of sieved soil using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. A negative control using empty extraction tubes was included to account for possible kit contaminants. Overall, 76 samples were processed: 10 developmental stages (including the control sites, s. above), 2 sampling dates – with the plots from 2015 and the freshly deposited material available only in June due to the ongoing restoration processes – 4 pseudo-replicates per developmental stage and an additional four negative controls.

DNA extracts were used to quantify 16S rRNA gene copy numbers as a proxy for the abundance of bacteria using real-time quantitative PCR (qPCR). In pre-experiments, potential inhibitions of the used polymerase were tested and the best PCR efficiency (mean: 1.55, SD: 0.02) obtained for 1:16 dilutions of the original extracts (data not shown). For quantification the 2× Takyon for SYBR Assay master mix (Kaneka Eurogentec S.A., Seraing, Belgium) was used following the manufacturer's instructions with an additional amount of 0.06% Bovine serum albumin (Sigma-Aldrich Corp., St. Louis, MO, USA). The primers FP 16S rDNA (5′-GGTAGTCYAYGCMSTAAACG-3′) and RP 16S rDNA (5′-GACARCCAT GCASCACCTG-3′) were used for amplification (Bach et al., 2002). Each sample was measured in three technical replicates on a 7300 real-time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR program consisted of a 3 min initial denaturation step, followed by 40 cycles (denaturation 10 s, 95 °C; annealing 20 s, 58 °C; elongation 45 s, 72 °C). Each reaction plate included a standard curve (s. below) and three negative PCR reactions. A melting curve analysis was performed to check for reaction specificity. The obtained initial fluorescence values per sample were converted into gene copy numbers per gram of dry soil using standard curves ($R^2 > 0.99$ for all reaction plates) from 10-fold dilutions of standards (16S rRNA gene of *Pseudomonas putida*) with known copy number ranging from 5.6×10^4 to 5.6×10^8 copies μl^{-1}. The real-time data were subsequently analysed in R using the package qpcR (Spiess, 2014) employing the mechanistic cm3 model (Carr and Moore, 2012), which minimises the effect of inhibitors and is insensitive to low PCR efficiencies, after baseline subtraction and normalisation. Kinetic outliers were removed automatically.

2.4. 16S rRNA gene library preparation and sequencing

DNA concentrations were measured on a fragment analyzer (Advanced Analytical Technologies, Inc., Ames, US-IA). The V1-V2 region of the bacterial 16S rRNA gene was amplified from 10 ng input DNA using the primers S-D-Bact-0008-a-S-16 (5′-AGAGTTTGATCMTGGC-3′) and S-D-Bact-0343-a-A-15 (5′-CTGCTGCCTYCCGTA-3′) (Klindworth et al., 2013). This region was chosen due to its better discriminative performance when compared to others (Johnson et al., 2019), e.g. compared to the V4 region recommended by the Earth Microbiome Project. PCR reactions were carried out in triplicate to compensate for random differences in amplification; in addition, a negative reaction control was included. The NEBNext High-Fidelity 2× PCR Master Mix (New England Biolabs, Ipswich, MA, USA) was used with an initial denaturation step of 5 min at 98 °C followed by 25 cycles (denaturation 10 s, 98 °C; annealing 30 s, 60 °C; elongation 30 s, 72 °C) and a final elongation time of 5 min at 72 °C. The amplification quality was analysed on a 1% agarose gel before pooling the triplicate reactions for purification using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The samples were further subjected to quality control and concentration measurement using a fragment analyzer (Agilent Technologies, Inc., Santa Clara, CA, US), checking for correct amplicon sizes and absence of primer dimers in the samples. Thereafter, 10 ng of amplified DNA per sample were indexed for multiplexed short-read sequencing using the Nextera XT Index Kit v2 (Illumina, Inc., San Diego, CA, USA). The according PCR program consisted of an initial denaturation step of 30 s at 98 °C followed by 8 cycles – denaturation 10 s, 98 °C; annealing 30 s, 55 °C; elongation 30 s, 72 °C – and a final elongation time of 5 min at 72 °C. Quality controls and concentration measurements of the indexed amplicons were analogous to the previous step. After diluting to 4 nM, the samples were pooled for sequencing on a MiSeq System (Illumina, Inc.) using the MiSeq Reagent Kit v3 (600 cycle) for paired end sequencing following the manufacturer's instructions. A final amount of 10 pmol of DNA was loaded, including 20% of PhiX (Illumina, Inc.) as a positive control.

2.5. Bioinformatic analysis

A detailed description of the bioinformatic and statistical procedures used for data analysis is available in the Supplementary material. A total of 7.67 million raw reads with 106,153 ± 23,915 reads (mean ± SD) per sample were generated. After quality filtering steps and contaminant removal 85.76% of the raw reads remained for further analysis. Sequences were de-noised using DADA2 and clustered to OTUs at 97% sequence similarity using MeShClust2 to prevent overestimation of α-diversity (Callahan et al., 2016; James and Girgis, 2018). After estimation of α-diversity and species evenness as Shannon diversity and Pielou's evenness index, respectively, the data set was filtered to contain only those OTUs present in at least two samples. This left 4985 OTUs from 6,489,334 reads (84.66% of total raw reads) with an average of

90,130 reads per sample (min: 46,858, max: 149,270) for further consideration. To correct for library size differences between the samples, the count data were subsampled to equal library sizes of 46,858 reads. Rarefaction plots showed a clear saturation of the number of OTUs at this amount of reads for all samples (Fig. S2).

Hierarchical clustering was performed on Bray-Curtis distances and clusters were visualised and tested for statistical significance using constrained analysis of principal coordinates (CAP). Bacterial phyla responding to restoration age were identified using linear mixed models (LMM). OTUs specific for restoration clusters, specific for the reference sites and the core bacterial community of all restored sites were identified by subsetting the data set accordingly. To benchmark the effect size of soil ageing after restoration, we compared the obtained data with the effect of seasonal dynamics on the bacterial community. To this end we calculated the average amount of OTUs present only at one sampling date per restoration cluster. This we compared to the average amount of OTUs specific for each restoration cluster irrespective of season. The abiotic factors exhibiting the largest changes between restoration clusters were found using robust one-way and two-way ANOVA procedures in combination with generalised η^2 effect sizes. Correlations of soil chemical parameters with the relative abundance of single OTUs were calculated as Spearman's rho.

2.6. Comparison to other data sets

We compared our results to three similar projects on opencast mining restoration sites. All of these used a 16S rRNA sequencing approach to describe bacterial soil community structure using the Illumina MiSeq platform. Study 1 investigated the factors influencing microbial succession following surface mining (Kane, 2019). The data set comprises the results from soils of four ages (2, 10, 15 and 32 years after reclamation) in West Virginia, USA. Soils were re-vegetated using mixed-grass leguminous species such as orchard grass (*Dactylis glomerata*), clover (e.g., *Trifolium spp.*), birdsfoot trefoil (*Lotus corniculatus*), and fescue grasses (*Festuca arundinacea*). The sequencing data are available from the SRA database, BioProject ID PRJNA529237. Study 2 contained data from a mining restoration chronosequence in Virginia, USA. The soils were 5, 10, 20 and 30 years of age and were re-forested using native hardwood tree species (Sun et al., 2017). From this data set, BioProject ID PRJNA324696, we chose samples only from May and July samplings for reasons of comparability to our data. Study 3 investigated the effect of opencast mining reclamation on the soil microbiome under wood and pasture vegetation in Mississippi, USA. The investigated sites were chosen at 1, 4, 8, 11 and 13 years after reclamation. The data is available under BioProject ID PRJNA529248. The data sets were processed analogous to our data as described above.

2.7. Accession numbers

Data on soil chemical properties are publicly available on the BonaRes Data Portal (https://datenzentrum.bonares.de) under the identifier 72ca6e98-5aab-4884-bf1b-56931482eb94. The nucleotide sequence data reported will be available in the SRA databases under the BioProject ID PRJNA454771.

3. Results

3.1. Bacterial abundance and diversity measures

The abundance of bacteria based on the quantification of 16S rRNA genes per gram of dry soil was significantly lower in the freshly deposited substrate compared to OS, which was unaffected by mining (Fig. 1). However, in the first ten years of the restoration process, the abundance increased and reached numbers comparable to the abundance in the original soil and appeared constant thereafter. Bacterial abundance was slightly higher in June compared to March at all developmental stages of restoration.

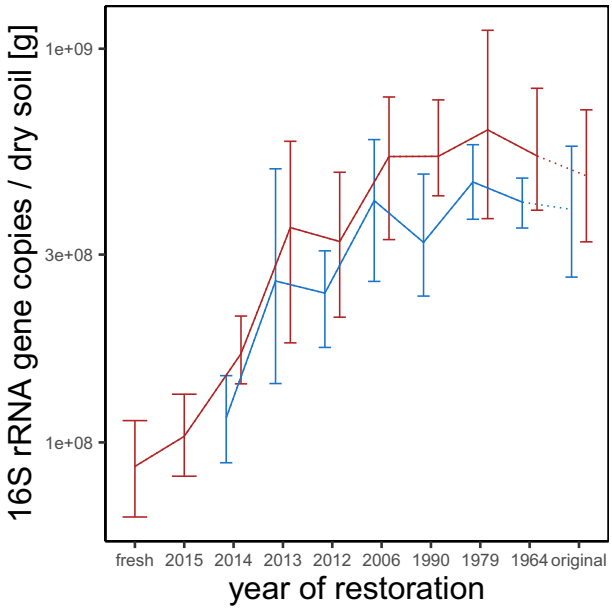


Fig. 1. 16S rRNA gene copies numbers determined by real-time quantitative PCR. Data presented in blue are from March samples, the ones in red are from June samples. "Fresh" refers to samples from the freshly deposited substrate, "original" to the original Luvisol. Error bars depict 95% confidence intervals of the four pseudo-replicates. Solid lines connect samples from the chronosequence, dotted lines serve for comparison to the original Luvisol.

Richness of bacterial OTUs at the reclaimed sites was comparable to the original soil already in samples from RS1 and even exceeded the original soil thereafter (Fig. 2). Concerning seasonal variation, we found significant differences between March and June samples only in the aged plots of RS3, which were reclaimed earlier than 1990.

Bacterial communities formed four clusters as determined by hierarchical clustering. These clusters corresponded to restoration years 2015–2013 (RS1), 2012–2006 (RS2 and early RS3), and 1990–1964 (late RS3). Surprisingly, the fourth cluster constituted the freshly deposited substrate as well as OS, which might be a consequence of mixing the original Luvisol with the substrate. CAP ordination was used to visualise the clustering and to check if the calculated clustering was statistically significant. The ordination depicted a total of 36.14% constrained variance and both the model ($p = 0.016$, Pseudo-$F_{3, 68} = 12.83$) and the first axis ($p = 0.015$, Pseudo-$F_{1, 68} = 22.90$) were significant (Fig. 3). To describe the soil conditions in those restoration age clusters defined via the bacterial community structure of the sites, we compared the soil chemical properties between those clusters statistically. Most important drivers were shifts in pH, total N content as well as C_{org} content in the soil samples of the different restoration stages. A complete overview of all parameters analysed is given in the Supplementary material (Table S2).

3.2. Identification of main bacterial responders to soil ageing and land use

From the ten most abundant bacterial phyla, seven changed significantly between the restoration stages; interestingly we found that none of them was influenced by the season (Table 1). OTUs assigned to Proteobacteria as well as Gemmatimonadetes decreased in relative abundance from RS1 to RS3, while those from Acidobacteria, Nitrospirae and Firmicutes increased at the same time. Cyanobacteria were found most abundant in samples from RS1. Comparing RS3 and OS, we observed a significantly lower relative abundance of OTUs assigned to Nitrospirae in samples derived from RS3, while we found a higher

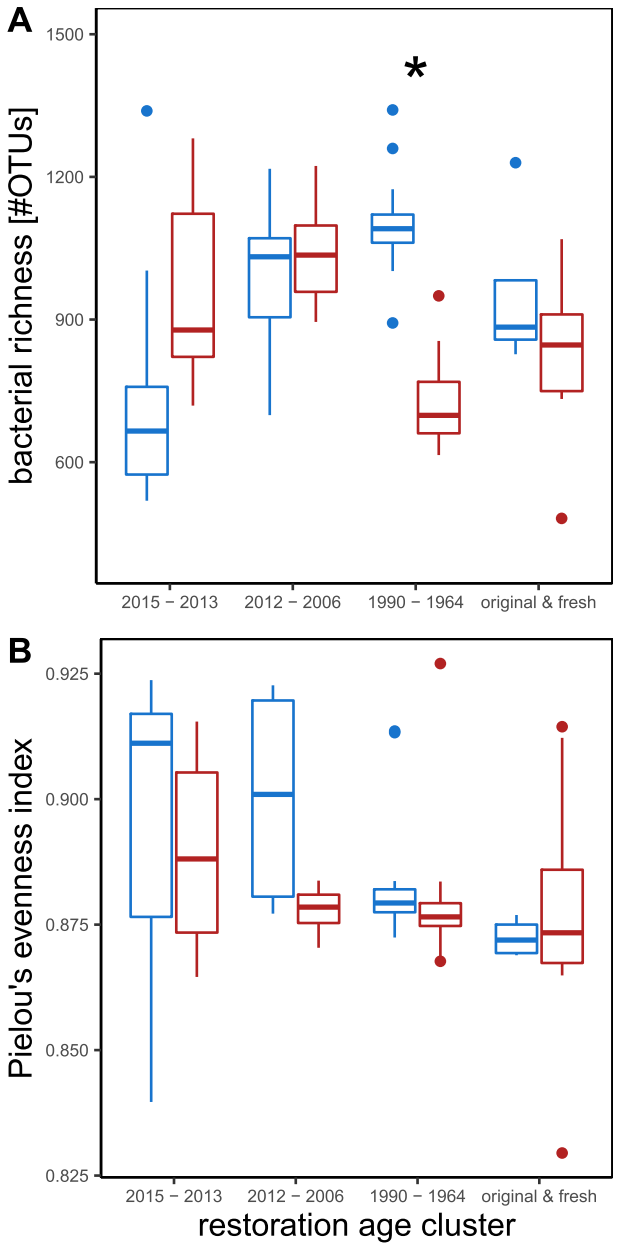


Fig. 2. Bacterial richness (A) and evenness (B) observed in the 16S rRNA gene libraries. Data presented in blue are from March samples, the ones in red are from June samples. "Fresh" refers to samples from the freshly deposited substrate, "original" to the original Luvisol. Dots depict outliers, asterisks denote significant differences between groups at a 5% significance level.

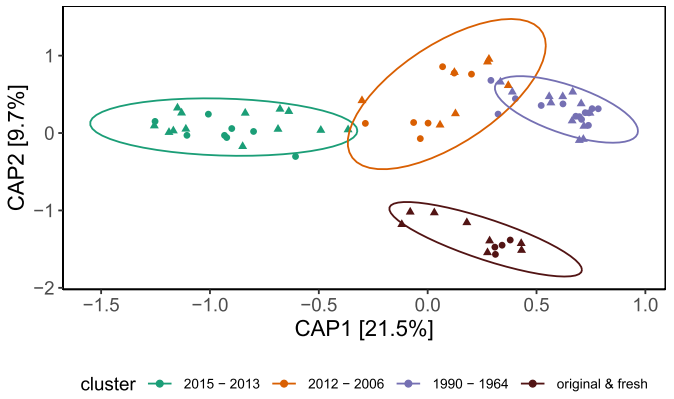


Fig. 3. CAP ordination of bacterial community structure. The calculated hierarchical clustering of the bacterial community was used as explanatory variable for the model. Clusters are represented in different colours. Circles represent samples from March, triangles represent samples from June sampling campaigns, respectively. Ellipses represent 95% confidence ellipses per age cluster.

relative abundance of OTUs which could be assigned to Latescibacteria in OS samples (Fig. 4).

In a second step, OTUs specific for the different restoration age clusters were identified. RS1, contained a total of 3197 OTUs, of which 672 (21.0%) were specific for that cluster. For RS2, only 315 (9.7%) specific OTUs were found, despite a comparable amount of 3255 OTUs in total. For samples from RS3, we found 487 OTUs out of 3042 (16.0%) being specific for these sites. To gain further insights into those specific OTUs, we compared their taxonomic assignments between the restoration stages.

While some genera appeared in all clusters – although with different OTUs – each cluster also contained a set of unique genera (cf. Table S3). At RS1, the most abundant unique OTUs were assigned to the genera *Chryseobacterium*, *Pedobacter*, *Dyadobacter* as well as three members of Cyanobacteria. In samples from RS2 fewer genera were specific for these sites. However, OTUs from *Actinoplanes*, *Rhodomicrobium* and *Aquicella* were identified to be specific for the intermediary age cluster. In the oldest reclaimed sites from RS3 the most abundant specific OTUs belonged to the families Peptostreptococcaceae, Desulfurellaceae as well as Streptomycetaceae. However, from these only the genera *Streptomyces* (Streptomycetaceae) and the uncultured group H16 (Desulfurellaceae) could be assigned to the genus level.

Due to the cultivation of the legume alfalfa during RS1, OTUs belonging to the order Rhizobiales were of special interest. Despite the fact that OTUs indicative for Rhizobiales appear in all clusters, the restoration clusters harbour a different composition of Rhizobiales. While for RS1 we identified specific OTUs from 11 genera of Rhizobiales (*Aquamicrobium*, *Devosia*, *Hansschlegelia*, *Hyphomicrobium*, *Labrys*, *Neorhizobium*, *Parvibaculum*, *Pedomicrobium*, *Prosthecomicrobium*, *Rhizobium* and *Rhizomicrobium*), RS3 only harboured four OTUs which could be linked to Rhizobiales (*Ancylobacter*, *Devosia*, *Nordella* and *Rhizomicrobium*) (Fig. S3).

Similar observations were also made for other taxa, including *Streptomyces*. We found 11 OTUs assigned to *Streptomyces* in our dataset (Fig. S4). While some OTUs increased in relative abundance from RS1 to RS3 (OTUs 4 and 8), others decrease over time in the restored sites (OTUs 2 and 3). In addition several *Streptomyces* OTUs exhibit clear preferences for certain restoration stages or ranges of stages (OTUs 1, 3, 5, 6, 9, 10 and 11). The correlation of single *Streptomyces* OTU with soil chemical properties indicated differing correlations of soil chemical properties and even closely related OTUs present predominantly in early restoration stages compared to those predominant in RS3 (Fig. 5).

OTUs which belonged to a core microbiome and were present at all restored sites consisted of 1622 OTUs and contained OTUs linked to the genera *Pseudarthrobacter*, *Acidibacter* and *Gaiella* as well as OTUs which could be assigned to the families Comamonadaceae and Nitrosomonadaceae (Table S3).

We found an average of 643 ± 277.8 (mean ± SD) OTUs to change between sampling dates. Restoration stages on average differed by 492 ± 178.5 OTUs. Within the most abundant season-specific OTUs we found Bacillales (*Bacillus* and *Paenibacillus*), Flavobacteriales (*Chryseobacterium* and *Flavobacterium*) and Cytophagales (*Dyadobacter*, *Hymenobacter* and *Adhaeribacter*) in all March samplings of all clusters, but never in June. However, the June samplings did not exhibit such commonalities (Table S3).

3.3. Comparison with other reclamation chronosequences

We subjected three publicly available data sets based on 16S rRNA sequencing data from reclamation chronosequences to the same data processing and analysis approach as presented for our data. In a first step, we compared the α-diversity based on Shannon indices in each

Table 1
Top ten most abundant phyla in the data set. Shown are p and F values and degrees of freedom for the overall linear mixed models per phylum as well as model estimates and p-values (in brackets) for planned contrasts and the random effect of the linear mixed models. The p-values for the overall effect of the age cluster were corrected using the Bonferroni method. Lines presented in boldface show significant differences between the 1990–1964 age cluster and the group containing the original Luvisol and the fresh substrate material. Asterisks denote a significant overall effect of the restoration age cluster on the abundance of the corresponding phylum. A graphical overview is given in Fig. 4.

Phylum	Overall model		Intercept	2015–2013 vs. others	2012–2006 vs. 1990–1964 and original & fresh	1990–1964 vs. original & fresh	Sampling date (random effect)
Proteobacteria	**<0.001***	**$F_{3,67.2} = 9.2$**	**36.0 (0.021)**	**−1.2 (<0.001)**	**−1.6 (0.002)**	**0.3 (0.710)**	**0.148**
Actinobacteria	0.004*	$F_{3,67.1} = 6.8$	19.0 (0.037)	0.3 (0.289)	1.4 (0.001)	−1.1 (0.115)	0.080
Acidobacteria	**<0.001***	**$F_{3,68.0} = 18.6$**	**14.6 (<0.001)**	**1.3 (<0.001)**	**1.2 (<0.001)**	**0.2 (0.742)**	**1.000**
Bacteroidetes	**<0.001***	**$F_{3,68.0} = 9.5$**	**9.1 (<0.001)**	**−0.3 (0.106)**	**−1.5 (<0.001)**	**−0.3 (0.501)**	**1.000**
Chloroflexi	**1.000**	**$F_{3,68.0} = 2.0$**	**6.9 (<0.001)**	**−0.1 (0.038)**	**0.0 (0.555)**	**0.1 (0.374)**	**1.000**
Gemmatimonadetes	0.062	$F_{3,68.0} = 4.5$	5.4 (<0.001)	−0.2 (0.002)	0.0 (0.926)	0.2 (0.199)	1.000
Nitrospirae	**<0.001***	**$F_{3,67.2} = 32.6$**	**2.1 (0.029)**	**0.3 (<0.001)**	**0.3 (<0.001)**	**0.2 (0.020)**	**0.391**
Latescibacteria	**<0.001***	**$F_{3,67.5} = 30.8$**	**0.8 (0.019)**	**0.1 (<0.001)**	**0.0 (0.198)**	**−0.2 (<0.001)**	**0.966**
Firmicutes	**<0.001***	**$F_{3,67.3} = 12.7$**	**0.6 (0.061)**	**0.1 (<0.001)**	**0.1 (0.005)**	**−0.1 (0.130)**	**0.547**
Cyanobacteria	0.356	$F_{3,68.0} = 3.0$	0.7 (0.001)	−0.2 (0.073)	0.2 (0.307)	0.6 (0.046)	1.000

site (Fig. S5). While bacterial α-diversity in studies 1 and 3 was comparable, data from study 2 exhibited the lowest diversity overall. Data from our study revealed on average the highest α-diversity. Except for study 2, α-diversity in all studies peaked at an intermediate restoration stage. However, timing of the restoration process was differing in all studies, thus an age-related definition of an intermediate restoration stage was not possible. For all studies we collected specific and core OTUs for the available site ages analogous to the results shown above for our data. Focussing on the early and late years after restoration, we searched for common patterns in the specific OTUs in each of the data sets. Most commonalities were found on the level of orders. For the early stages, Cytophagales and Flavobacteriales were typical for the first up to ten years after restoration. Further, in three out of four chronosequences we found OTUs of Cyanobacteria specific for the first five years. Only for study 1 no cyanobacterial OTUs were detected at early time points of restoration. Later stages were characterised by Desulfurellales, for all studies except study 1. Frankiales were found in late stages of studies 1, 2 and 3, but not in our sites. In our sites and in study 2, we found Clostridiales in the oldest sites. The core or bacterial community of the restored sites had several members in common between all studies included into our meta-analysis. Besides members of the Rhizobiales, we found Blastocatellales (subgroup 4), Sphingobacteriales (family Chitinophagaceae), Burkholderiales (family Comamonadaceae) and Nitrosomonadales (family Nitrosomonadaceae) to consistently contribute to the core community of restored mining sites.

4. Discussion

4.1. Chemical and biological developments after restoration

Anthropogenic activity is one of the strongest known factors to influence soil ecosystems (Gupta et al., 2017). However, while the underlying concepts and mechanisms of anthropogenic impact are well established for macroecology, the consequences of human action for microecology are still poorly understood. Biodiversity and community composition are key parameters in determining multiple ecosystem functions fulfilled by soil (Wagg et al., 2014). To this end, our first study aim was to investigate how anthropogenic intervention drives the development of soil bacterial communities. We studied a chronosequence of restored opencast mining sites. We analysed the development of soil bacterial communities over a 52-year period. Early studies on the restoration of mining sites mainly focussed on single parameters like microbial biomass and activity, or potential soil enzyme activities (Insam and Domsch, 1988; Bentham et al., 1992; Emmerling et al., 2000). All of these however, tend to recover within a few years after the soil restoration, if properties of the used parent material allow it (Insam and Domsch, 1988; Reichel et al., 2017). Our observations on bacterial abundance and α-diversity are in line with these findings, indicating a quick recovery to the levels described for the original soil after a few years. However, soil chemical properties exhibited marked differences between the restoration stages as well as in comparison to OS. Compared to the original Luvisol, the soil properties after restoration differed even after the substrate aged for 52 years and even though the substrate was mixed with the Luvisol at the beginning of the restoration procedure. Most importantly, pH, organic carbon and total N contents show that life conditions for soil biota are different in each restoration stage and different form undisturbed conditions. After analysing the bacterial community composition from each soil age class, we were able to identify three clearly distinct clusters of bacterial communities (Fig. 3). The reference sites formed a fourth cluster. An exemplary correlation analysis for *Streptomyces* OTUs further underlines the effect of soil chemical properties on individual taxa, but also demonstrates that the comparable soil properties affects closely related species obviously in different ways (Fig. 5). The clustering pattern corresponded well to the timing of changes in site management during the restoration procedure. The first turnover from alfalfa cultivation to agricultural use constitutes a significant change and was thus followed by a clear and quick turnover of the bacterial community composition. However, the second stage transition from one agricultural management regime to another was likely a less pronounced one. This was reflected by a longer turnover phase with the earliest plots of RS3 being still more similar to the plots of RS2 rather than to older plots from RS3. To gain a better understanding of the extent of these changes we created an internal baseline using the seasonal changes between our two sampling dates. We found that changes between restoration stages had a similar extent as those of seasonal changes at our sites. Since it has previously been shown that the season has a major impact on bacterial communities in agricultural soil (Lan et al., 2018), this demonstrates the significance of land management regimes for the bacterial soil community.

4.2. Characteristics of bacterial communities over time

We further characterised the restoration stages by studying their bacterial community profiles. On a high taxonomic level, nitrifiers of the phylum Nitrospirae were affected the most across the different restoration stages. As these microbes require specific niches to avoid competition by fast growing heterotrophs, it is likely that the restoration processes generated different niches compared to the original soil in early restoration stages, thereby reducing the competitiveness of Nitrospirae towards other microbes. Overall a strong increase of Firmicutes and other (facultative) anaerobic microbes was found from RS1 to RS3. This might that the oxygen supply and the associated redox conditions in the early restored soils were different compared to OS, reflecting the sticky texture we observed in these soils.

Further insights were generated by analysing specific OTUs of each restoration stage. In RS1, we identified OTUs from the genera

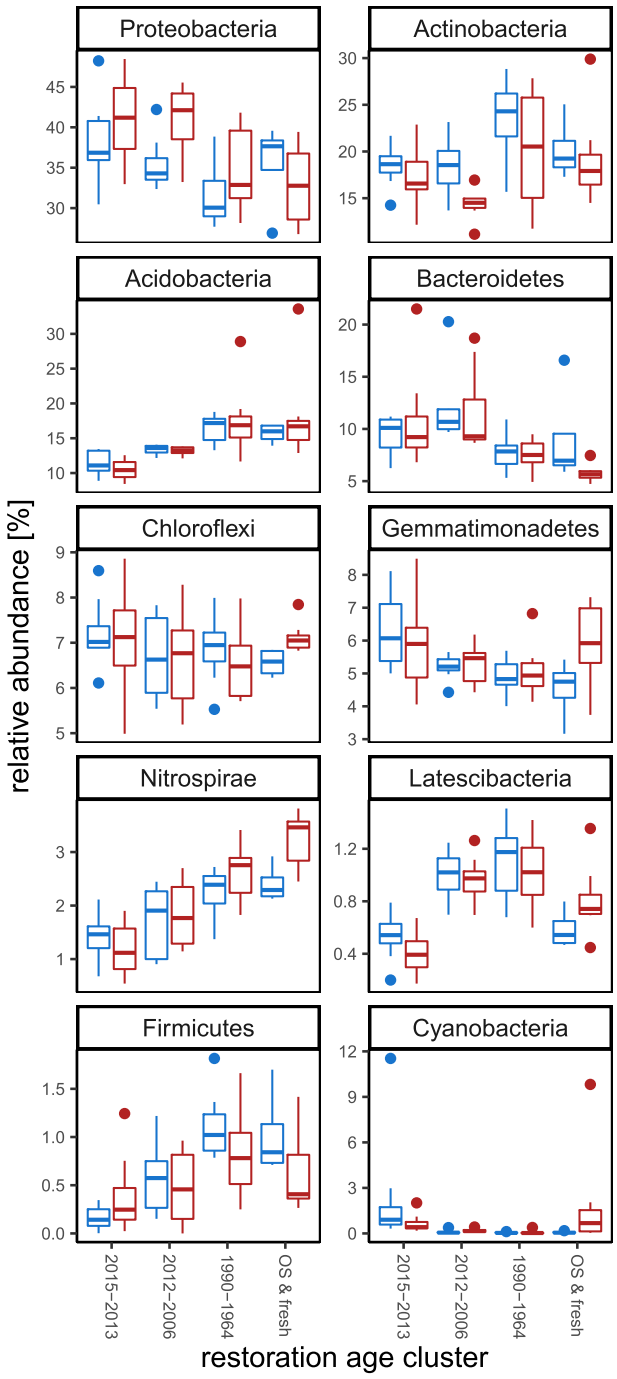
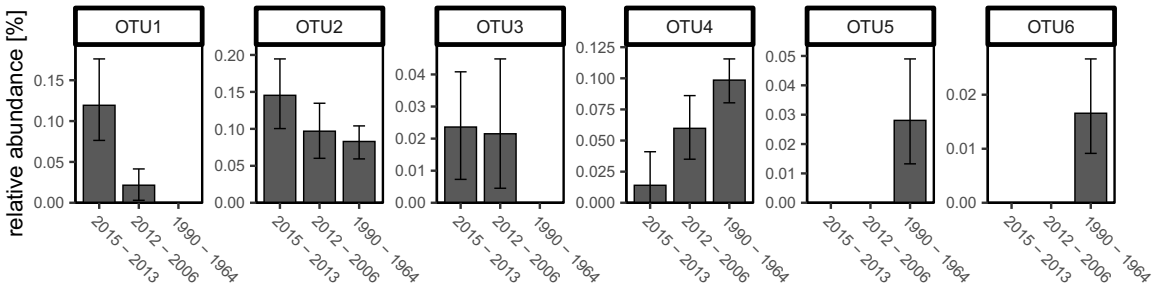


Fig. 4. Top 10 most abundant bacterial phyla in the data set. The presented values are the relative abundances of all OTUs assigned to the respective phylum in a sample. Data presented in blue are from March samples, the ones in red are from June samples.

Chryseobacterium, *Dyadobacter* and *Pedobacter* as characteristic members of bacterial communities. All of these are often found in soil, but also in other habitats. *Dyadobacter* is often associated with plants while it was found to hydrolyse neither cellulose nor starch (Chelius and Triplett, 2000). As the organisms within these genera all occupy a wide range of habitats, which indicates their flexibility to adapt to changing environmental conditions. One obvious link to the chosen restoration strategy is the high diversity of Rhizobiales found in RS1 compared to RS3. We attribute this to cultivating of the legume alfalfa during early RS1, which was performed to enhance microbial life in soil and to increase the nutrient level in the young substrates. In the second cluster of bacterial communities, representing RS2 and early RS3 conditions, we observed characteristic OTUs from *Actinoplanes*, *Rhodomicrobium* and *Aquicella*. *Actinoplanes* are known to be slow-growing and prefer habitats with periodic wetting and drying events (Vobis, 2006). This is in line with our observations of the restored sites forming hard crusts when drying during summer. *Aquicella* has a growth optimum in neutral pH, which also meets the conditions of our restored sites (Santos et al., 2003). *Rhodomicrobium* are photoheterotrophic organisms. Since for the conversion from RS1 to RS2 the soil is tilled, these photoheterotrophs might have been favoured by the restoration management at this stage. Late soils of RS3 harboured OTUs from strictly anaerobic as well as aerobic taxa. For the anaerobic ones we found Peptostreptococcaceae, a family within the order Clostridiales, which grows on proteinaceous substrates and carbohydrates (Slobodkin, 2014). We also found the undescribed genus H16 from the family Desulfurellaceae, all known members of which use sulphur or thiosulfate as an electron acceptor (Kuever et al., 2015). A possible explanation for this observation might be the increasing sulphur content over time in the restored sites, possibly caused by fertilisation with ammonium thiosulphate together with the formation of soil aggregates with reduced oxygen concentrations in the interior. Lastly, OTUs from the aerobic genus *Streptomyces* characterised RS3. Members of this genus are widely known as decomposers growing on decaying vegetation or recalcitrant organic matter in soil. Also their hyphal growth and rich secondary metabolism are well-known. Since *Streptomyces* is a very large genus with more than 500 known species, we suspected not all species present in our soils to react in the same way to changes in land management. We thus provided further insights beyond the RS3-specific OTUs and indeed found different occurrence patterns of the different *Streptomyces* OTUs. These might have been influenced the most by the changes of soil chemical conditions over time in the ageing substrate. However, other *Streptomyces* OTUs exhibited significant abundance changes at those points in time when land management changes occurred according to the restoration procedure. This is a clear indication of human influence on site development and bacterial community composition. Given the importance of these organisms for the decomposition of organic matter and their ability to produce multiple antibiotic substances, this reveals one way of how land management interferes with soil functions.

4.3. General trends in restored mining sites

To generalise our results beyond the applied restoration practices, in order to find general trends during site development after restoration, we analysed three published datasets of chronosequences from restored mining sites in the same way as our own dataset. Some of the observations we made in our restoration sites, could be confirmed for other restored mining sites. In all the studies we identified Cytophagales in the early stages after restoration. These organisms are organotrophs, degrading biomacromolecules such as proteins, chitin, pectin or cellulose. As such the Cytophagales play an important role for the turnover of organic matter in nature, especially in soils at neutral pH (Reichenbach, 1992). Hence, during the restoration of mining sites, Cytophagales might be an important part of the decomposer community of young developing sites. As another characteristic group of early restoration stages we identified the order Flavobacteriales, of which we observed *Chryseobacterium* in our sites. Since *Chryseobacterium* shows proteolytic activity and most Flavobacteriales require organic compounds for growth (Vandamme et al., 1994; Bernardet, 2015), this group might be another constituent of the decomposing community. Cyanobacteria were found as another typical early coloniser across the studies. Early restoration sites with their large proportion of bare soil tend to offer good growth conditions for these phototrophs. This also conforms with our observation that early restoration sites had only a minimum plant cover until the alfalfa seedlings established after several months. Also, before cultivation of alfalfa, the substrate is first left to settle, before larger proportions of land are taken into agricultural management.



	pH	NH4+	total N	C_org	P_CAL	P	K_CAL
OTU1	**0.75**	**0.48**	**-0.71**	**-0.58**	**-0.62**	**-0.54**	**-0.65**
OTU2	0.33	0.19	-0.37	-0.31	-0.34	-0.36	-0.31
OTU3	0.35	0.27	-0.28	-0.23	-0.27	**-0.45**	-0.27
OTU4	**-0.61**	**-0.39**	**0.54**	**0.46**	**0.39**	**0.42**	**0.42**
OTU5	-0.17	-0.31	0.32	**0.41**	**0.49**	**0.49**	**0.50**
OTU6	-0.37	**-0.40**	0.37	**0.42**	**0.42**	0.40	0.34

Fig. 5. Upper part: relative abundance of OTUs assigned to the genus *Streptomyces* in the age clusters of the restored sites. Bars represent means, error bars bootstrapped 95% confidence intervals. Lower part: Spearman's rho correlation coefficients of the presented abundance data with soil chemical properties. pH: pH value, NH4+: ammonium content, total N: total N content, Corg: organic carbon content, P_CAL: plant-available phosphorous content, P: total P content, K_CAL: plant-available potassium content. Numbers in bold face represent significant correlations on a 5% significance level.

At late stages across all studies, we identified OTUs assigned to Frankiales, Desulfurellales as well as Clostridiales. While Frankiales specialised to remarkably diverse ecological niches (Normand and Fernandez, 2020), some of them are symbiotic nitrogen fixers. The fact that we identified Frankiales in all published datasets but not in our own sites, might be connected to the type of landscape restoration applied in each case. While our sites were turned into agricultural land, with alfalfa, a legume with forms symbiotic interactions with rhizobia, as initial coloniser, the studies which were used for comparison were revegetated with grassland or forest. As such, Frankiales likely find suitable niches only in these undisturbed conditions resembling a natural vegetation. In all datasets except for study 1 we found Desulfurellales in plots older than 10 years. As mentioned above, at least in our sites this corresponded with an increasing sulphur content over time in the restored soils. Besides that, these organisms were previously reported to contribute significantly to the abundance of the nifH genes in soil and might fix nitrogen in old restored soils (Sumerta et al., 2020). Clostridiales as an order are too diverse for a straightforward interpretation. Further, at the family we found organisms utilizing proteins and carbohydrates as energy sources (Peptostreptococcaceae, s. above), but also groups harbouring typical gut bacteria (Ruminococcaceae, Christensenellaceae; Morotomi et al., 2011). All of these are anaerobic organisms, suggesting these soils to possess corresponding niches, most likely aggregates. Altogether, the Clostridiales OTUs we found are possibly part of the decomposing community. Finally, we found a number of bacterial core families in all the datasets we analysed. These were Chitinophagaceae, Comamonadaceae, Nitrosomonadaceae as well as members of the orders Rhizobiales and Blastocatellales. These are likely to represent either very generalist lifestyles or organisms, which provide key services to the soil ecosystem. For the latter, the nitrifying bacteria of the Nitrosomonadaceae family can be taken as an example.

4.4. Conclusions

In conclusion, our study provides detailed insights into how anthropogenic action during ecological restoration alters bacterial communities in soil. Land management had a clear impact on the overall development of community composition and favoured different bacterial taxa during the restoration process. Overall two types of responses of bacteria were observed in our study: (1) General succession pattern as a result of soil formation and the increase in soil nutrient content as well as specific niches e.g. for bacteria favouring anoxic conditions. This included Peptostreptococcaceae, Desulfurellaceae and Streptomycetaceae, which were found in all chronosequences analysed increased relative abundance values at later time points of the restoration process compared to early time points of restoration. (2) Taxa that specifically responding to the type of ecological restoration applied. This included in our case mostly Rhizobia which were increased in relative abundance also compared to the other chronosequences analysed as a matter of the cultivation of alfalfa. Over all our data however indicates that also after 52 years of restoration the original bacterial community composition has not been completely restored.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.140955.

CRediT authorship contribution statement

Christoph A.O. Schmid: Methodology, Validation, Writing - original draft, Writing - review & editing. **Rüdiger Reichel:** Methodology, Resources, Investigation, Writing - original draft, Writing - review & editing. **Peter Schröder:** Supervision, Writing - original draft, Writing - review & editing. **Nicolas Brüggemann:** Conceptualization, Funding acquisition, Writing - original draft, Writing - review & editing. **Michael Schloter:** Conceptualization, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors certify that there are no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or

non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Appendix II: Manuscript M2

Organic amendments in a long-term field trial – consequences for the bulk soil bacterial community as revealed by network analysis

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Keywords

microbial interactions, organic amendments, manure, straw, 16S amplicon sequencing, long-term experiment

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The nucleotide sequence data reported are available in the SRA databases under the BioProject ID PRJNA388309.

Abstract

This study intended to elucidate the long-term effects of organic soil amendments on bacterial co-occurrence in bulk soil with and without addition of mineral fertiliser. Previous research mostly neglected the bacterial co-occurrence structure and focussed mainly on the parameters species diversity and abundance changes of species. Here we present a systematic comparison of two frequently used soil amendments, manure and straw, with regard to their impact on bacterial co-occurrence in a long-term field trial in Speyer, Germany. The approach involved 16S amplicon sequencing in combination with a bacterial network analysis, comparing the different fertiliser regimes. The results show an increase of bacterial diversity as well as an accumulation of bacteria of the order Bacillales in plots fertilised with manure compared to a control treatment. In the straw-amended plots neither an increase in diversity was found nor were indicative species detectable. Furthermore, network analysis revealed a clear impact of mineral fertiliser addition on bacterial co-occurrence structure. Most importantly, both organic amendments increased network complexity irrespective of mineral fertilisation regime. At the same time, the effects of manure and straw exhibited differences that might be explained by differences in their nutritional / chemical contents. It is concluded that bacterial interactions are a crucial parameter for the assessment of amendment effects regarding soil health and sustainability.

Introduction

Following the demand of an increasing world population for food, agriculture had to face the challenge of maximising yields under the constraints of reduced availability of sites with high soil quality, during the last centuries. With the implementation of the Haber-Bosch process into agroindustry, synthetic fertilisers became the tool of choice to reach this goal. Initially, the awareness of potential environmental damages associated with the excessive use of mineral fertilisers in practice was neglected [1, 2]. However, today we are aware that abandoning traditional soil amendments like manure, compost and plant residues is detrimental for soil as well as plant health and finally soil fertility [3-5]. Much research has been done to elucidate the effects of mineral fertilisers on soil organisms [6-8]. A frequent result of these studies is a decreasing diversity mainly of soil microbes, commonly linked to decreased soil fertility. In contrast, the application of organic soil amendments resulted in positive effects and increased bacterial diversity as well as plant health [9-11]. In this respect, manure has been identified as a strong mitigator of detrimental effects from mineral fertilisation, while plant residues seem to be much less effective [12, 13]. Despite that, manure application on arable land might be critical due to an enrichment of antibiotic resistance genes [14-17].

In the long term it has been considered that organic amendments also influence soil quality positively [18]. Thus recent approaches in agriculture do not only rely on a combined use of organic and mineral fertilisers [19, 20], but also include the application of additional single organic amendments every 3 – 5 years with the aim to restore soil quality [21]. To address potential effects of such approaches, however, long term trials are needed, as time spans needed to improve soil quality are long. Furthermore, time-series are needed as especially α-diversity is known to change over the course of the year [22]. Thus, data on the influence of additional single organic amendment applications on the soil microbial community, which is one of the major drivers for soil quality, is rare.

Most amendments introduce complex, often polymeric, organic substances into the soil, that are degraded and metabolised by specialised microorganisms. Lignin, cellulose and hemicellulose

constitute major portions of the material introduced for amendment [23]. Given the complexity of these compounds, their biological degradation requires several specialised enzymes [24-26]. It has been observed that for an efficient degradation of cellulose more than one bacterial species is necessary [27, 28]. Stable cellulose-degrading communities even harbour species that do not themselves degrade cellulose, but facilitate degradation by maintaining favourable oxygen and pH conditions [27, 29]. It is therefore conceivable, that when assessing the fertility of amended soils, bacterial diversity is only one side of the coin, while an investigation on how organisms interact is the second.

In this study we present results obtained from a long-term experimental field study in Speyer, Germany, where additional single organic amendment applications were implemented into different mineral fertiliser regimes. Organic materials based on both horse manure and plant residues were used and effects were compared to controls where only mineral fertiliser was applied. We used a molecular barcoding approach to assess bacterial diversity patterns based on DNA directly extracted from bulk soil, amplification of the 16S rRNA gene by PCR and high throughput sequencing of the obtained amplicons. We analysed samples at different time points throughout the vegetation period. Besides ✔- and ß-diversity we assessed novel co-occurrence networks, and we investigated the stimulation of such co-occurrence patterns by complex organic materials. Furthermore the difference in co-occurrence patterns between plots with differing organic amendments (horse manure vs. plant residues) was elucidated.

Materials and Methods

Experimental layout

The present study was conducted at the International Organic Nitrogen Fertilisation Trial (IOSDV), Rinkenbergerhof, Speyer, Germany [30, 31]. The plots for this long-term trial were established in 1983. Each plot has a size of 7.5 x 6 m. The trial investigates two factors, namely the use of different organic soil amendments and increasing mineral N application rates. As for the organic amendments, horse manure and plant residues are applied, along with untreated control plots. Previously stored horse manure is applied every third year in autumn after winter barley had been

grown (cf. crop rotation in next section) at a concentration of 300 dt FM ha^{-1}. Plant residues are cereal straw or sugar beet leaves that are leftovers from each harvest (2015: 10.2 dt DM ha^{-1} barley straw) as well as cut cover crops. Hence, the main difference between the amendment managements is the source of the amendment material, i.e. animal manure vs. vegetable biomass. As for mineral N application rates, these are applied in five levels reaching from 0 to 240 kg ha^{-1} (200 for winter barley). Each combination of organic amendment and mineral N application rate is replicated three times in the field. A complete field plan is available in Online Resource 1.

Agricultural management of field sites

The applied crop rotation includes sugar beet, winter wheat and winter barley. Additionally, *Raphanus sativus* var. *oleiformis* is used as a cover crop every third year after winter barley in the plant residue treatment only and mulched before tilling. All plots received a basal dressing with a total of 35 kg P ha^{-1}, 232 kg K ha^{-1}, 66 kg Mg ha^{-1} and 1.6 kg Mn ha^{-1} in spring (P, K, Mg) or autumn (Mn). During the sampling campaigns for this study mineral N was applied twice in May '16. Tillage (plough) has been done equally on each plot up to a depth of 30 cm in November.

Site characteristics

The soil has been characterised as a Cambisol with an average pH of 6 – 6.5 containing 8.9 % clay, 48.3 % silt and 42.8 % sand. Total N ranged from 0.06 % to 0.1 %, and total C from 0.5 % to 1.0 %. In non-amended control plots the agricultural practices that were described above, led to a reduction of organic carbon contents from originally 0.83 % C to 0.60 – 0.70 % C in the 0 - 30 cm soil layer. Plots amended with either horse manure (0.74 – 0.85 % C) or plant residues (0.74 – 0.82 % C), however, were close to the original value. Similarly, the organic N content of originally 0.07 % was found reduced to 0.053 – 0.065 % N in the control plots, while almost unchanged in the horse manure (0.066 – 0.077 % N) and plant residue (0.068 – 0.076 % N) treatments. The climate at the experimental farm is characterised by a mean annual temperature of 10.0 °C, a mean annual precipitation of 593 mm and an annual sunshine duration of 1,441 h.

Soil sampling procedure

In the frame of this study plots from two levels of mineral N fertilisation were sampled, i.e. “without” (0 kg N ha^{-1}) and “with” (120 + 60 kg N ha^{-1} to sugar beet, 90 + 50 + 40 kg N ha^{-1} to winter wheat and 80 + 30 + 40 kg N ha^{-1} to winter barley). Since the selected plots included all organic amendment variants, this corresponds to a total of 18 plots sampled in four temporal replicates. As the study aimed at long term changes in bacterial communities, sampling campaigns were performed either prior to or well after management activities, to avoid effects caused by heavy disturbances. They took place in September '15 (fallow ground with volunteer crops / cover crop; before manure application), February '16 (fallow ground with volunteer crops / cover crop), June '16 (sugar beet) and November '16 (sugar beet). Thus we covered the complete vegetation period of sugar beet. Sampling was performed with a core sampler up to a depth of 30 cm. From each plot 12 soil cores were taken and thoroughly mixed by hand. A sub-sample of 100 g was taken for bacterial community analysis, sieved to 2 mm, and frozen on dry ice while immediately on the field. The remaining soil material was stored at 4 °C and subsequently used for the analysis of soil chemical properties (ammonia, nitrate and dissolved organic carbon) according to VDLUFA [32]. Samples from replicated plots were treated as true replicates.

16S library preparation

500 mg of soil were extracted using the NucleoSpin Soil Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's protocol. A negative control was done using empty extraction tubes for every sampling date. The DNA extract was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, US-MA). The V1-V2 region of the 16S rRNA gene was amplified using the primers S-D-Bact-0008-a-S-16 (5′-AGAGTTTGATCMTGGC-3′) and S-D-Bact-0343-a-A-15 (5'-CTGCTGCCTYCCGTA-3') described by [33]. PCRs were carried out in triplicate using the NEBNext High-Fidelity 2X PCR Master Mix (New England Biolabs, Ipswich, US-MA) with 10 ng input DNA. The PCR program included an initial denaturation step of 5 min at 98 °C followed by 25 cycles – denaturation 10 s 98 °C; annealing 30 s 60 °C; elongation 30 s 72 °C – and a final elongation time of 5 min at 72 °C. PCR

amplicons were checked on 1 % agarose gels. For each sample three independent PCR reactions were performed and pooled. Purification of PCR products was performed using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) and analysed on a 2100 Bioanalyzer Instrument (Agilent Technologies, Inc., Santa Clara, US-CA). 10 ng of amplified DNA per sample were indexed using the Nextera XT Index Kit v2 (Illumina Inc., San Diego, US-CA) for multiplexed short-read sequencing. The PCR program used included an initial denaturation step of 30 s at 98 °C followed by 8 cycles – denaturation 10 s 98 °C; annealing 30 s 55 °C; elongation 30 s 72 °C – and a final elongation time of 5 min at 72 °C. The resulting amplicons were checked as described above, diluted to 4 nM and pooled equimolar for sequencing on a MiSeq System (Illumina Inc., San Diego, US-CA) using the MiSeq Reagent Kit v3 (600 cycle) for paired end sequencing following the manufacturer's instructions. A final amount of 11 pM DNA was loaded. As a positive control PhiX (Illumina Inc., San Diego, US-CA) was used as a spike-in.

Processing of the sequencing data

De-multiplexed raw data from the MiSeq system was subjected to primer and adapter removal using the software AdapterRemoval V. 2.1.7 [34]. Subsequently, reads associated to PhiX were excluded and forward and reverse reads were merged using DeconSeq V. 0.4.3 [35]. For further data processing the QIIME pipeline V. 1.9.1 was used [36]. Prior to 97 % OTU clustering, the reads were filtered at a Phred quality score of 3; for downstream analyses only merged reads within a size range of 300 to 400 bp were processed further. For taxonomic assignments the RDP classifier V. 2.2 [37] retrained with the GreenGenes database V. 13.8 [38] was used. Rare sequences with a relative abundance below 0.005 % were filtered out. Finally, OTUs present in either negative extraction or negative PCR controls (0.3 % of reads) as well as chloroplast sequences were filtered out from the data set.

Statistical data analysis

Sequence data were imported to R V. 3.3.2 [39] using the phyloseq package V. 1.19.1 [40]. Plotting was carried out using the package ggplot2 [41]. Shannon diversity was computed from unfiltered OTU tables as recommended in the phyloseq documentation. Species evenness was

computed as Pielou's evenness index. Soil chemical parameters, soil DNA content, alpha diversity and taxa abundances were compared between treatments using a Bayesian model equivalent to a linear mixed model with time as a random factor. Long-term average crop yield data were supplied by the Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer, Germany. Yields between 2005 and 2016, i.e. four complete crop rotations, were compared using a Bayesian two-way ANOVA-like procedure [42]. For our dataset we considered Bayesian approaches more suitable than frequentist methods, as these are more flexibly adjustable to the data structure while still producing valid results even at low numbers of replicates. One experimental plot (treatment combination: mineral fertilised, without organic amendment) proved to be an extreme outlier in crop yield, DNA content, alpha diversity and community composition and was excluded from further analyses. For the production of a representative sample of the posterior distribution, Markov Chain Monte Carlo (MCMC) sampling was performed using the software packages JAGS V. 3.2.0 [43] and Stan V. 2.10.0 [44] as well as utility functions provided by Kruschke [42]. Four independent chains were sampled for 2,000,000 steps after a burn-in and an adaption period of 2,000 and 1,000 steps, respectively. Thinning was set to every 10^{th} step. Chain convergence was checked using trace plots to ensure that all chains came to the same result and none of them got stuck in a local maximum. Autocorrelation within the MCMC chain was assessed by the effective sample size (ESS) aiming at a lower limit of 10,000 for the relevant parameters, thereby ensuring that a truly representative sample of the posterior distribution was produced. In the following we will use the common notation of Bayesian statistics and use the term credibility rather than significance despite their similarity in interpretation. 95 % highest density intervals (HDI), rather than Bayesian p values or Bayes factors, served for checking the credibility of observed differences, as they provide more information about the posterior distribution and are generally more robust than Bayesian p values or Bayes factors (cf. [42]). A 95 % HDI for a mean difference was produced by calculating the difference at every step of the MCMC chain, thus generating a posterior distribution of the mean difference. The 95 % HDI then is the interval that contains the values with the 95 % highest posterior probabilities. Finally, an observed difference was considered credible, when its 95 % HDI did not include zero, i.e. a difference of zero had low posterior probability.

Ordination analysis

Beta diversity analysis was performed based on generalised UniFrac distances [45] of 97 % OTUs using partial constrained analysis of principal coordinates (CAP) as implemented in the capscale function from the vegan package V. 2.4-1 [46]. In order to assess exclusively the influence of fertiliser regimes on beta diversity, the soil parameters water content, pH, contents of ammonium, nitrate, mineral sulphur, phosphorus, potassium and magnesium, and the sampling time were partialled out in CAP. All of these proved to be significant drivers of microbiome structure in an earlier analysis step on a 5 % significance level. Subsequently, a permutation test (n = 999) was carried out to assess significance of the factors mineral and organic fertilisation using the date of the sampling as strata for permutations to correct for repeated measurements. The ordination was plotted as a split plot with 95 % confidence ellipses for the organic fertilisation treatments in the sample plot. OTUs were plotted using their annotated taxonomical order, reducing overlap for readability.

Identification of main responders

OTUs indicative of each organic amendment treatment were identified by means of Bayesian predictive modelling. For this purpose the BioMiCo algorithm [47] was employed with a delay of 5,000 steps. Two thirds of all samples were used for training and one third for testing the algorithm. Multiple runs were conducted in order to ensure that local maxima were avoided. OTU assemblages indicative of a treatment were defined as those that were present in the top 95 % posterior distribution of a single treatment only. The twenty OTUs with highest posterior probability in each assemblage were chosen and their absolute abundances plotted in a heatmap.

Computation of co-occurrence networks

Bacterial co-occurrence analysis was computed in the MetaMIS software V. 1.02 with default settings [48]. One network per treatment combination was calculated in order to compare treatment effects. The input data for the visual comparison were mean abundances over the three field replicates per time point collapsed to taxonomic families. For a statistical evaluation of network topology networks were computed for each plot and the resulting consensus networks exported to

Gephi V. 0.9.1 [49]. Average degree, average clustering coefficient and average path length were computed and compared statistically using a Bayesian two-way ANOVA-like procedure.

Results

Crop yields

Yield increases due to organic amendments depend on the planted crop as well as soil characteristics [50]. A more detailed analysis of crop yields is presented in Online Resource 1. Dry mass yields increased credibly for all crops when mineral N was supplied to plots (sugar beet: 114.2 dt ha^{-1}, winter wheat: 49.3 dt ha^{-1}, winter barley: 42.4 dt ha^{-1}) as compared to non-N-fertilised control plots (s.b.: 61.2 dt ha^{-1}, w.w.: 20.1 dt ha^{-1}, w.b.: 16.4 dt ha^{-1}). The organic amendments exerted highest influence on the sugar beet yields. However, their effect is most pronounced in the non-N-fertilised plots, where addition of any amendment credibly increased yield (95 % HDI: [12.2, 56.8]) compared to control conditions. When combined with mineral N the overall amendment effects are diminished (HDI [-8.2, 42.2]) and only plant residues increase sugar beet dry mass yield credibly compared to the control (HDI: [4.4, 60.2]).

Abiotic soil parameters

Gravimetric water content of the soil differed only by sampling date, but not by treatment. Potassium contents in soil were credibly higher in plots fertilised with either organic amendment compared to the control (HDIs: manure [-56.6; -15.2], plant residues [-55.3; -15.7]), while the manure and plant residue treatment were at the same level (HDI [-16.7; 16.9]). All other analytes did not show differences due to organic amendments, but only to mineral N additions. Results of the soil chemical analysis are summarised in tabular form in Online Resource 2. Compared to the control treatment without organic amendments, soil DNA was found to be elevated in both the manure and the plant residue treatment (95 % HDI none vs. manure [-3.1; -1.6]; none vs. residues [-3.3; -1.8]; Online Resource 1). No credible difference was found between applied amendments (HDI [-0.9; 0.5]). Treatments without mineral fertilisation showed an evident, yet non-credible increase in soil DNA content when compared to fertilised plots (HDI [-0.01; 0.96]).

Sequencing data

A total of 7.2 million reads were obtained from sequencing of which on average 96.0 % were retained after decontamination and merging of reads, 88.4 % after removal of low-quality reads and application of length filters, 66.5 % after OTU calling and taxonomic assignments and 49.8 % (3.6 million) after application of abundance filters. The final OTU table contained on average 50,223 reads per sample with a minimum of 24,452 and a maximum of 84,406 reads. This resulted in overall 3,282 OTUs (97 % identity). Taking account of the recent discussion in scientific literature concerning normalisation methods for unequal read counts [51, 52], alpha and beta diversity analyses were performed on non-normalised, rarefied and relative abundance data. We found no evidence for library size artefacts. Following the ideas of McMurdie & Holmes [51] we therefore present results based on the full, non-rarefied data. All samples show a sufficient coverage of the bacterial community as confirmed by rarefaction curves (Online Resource 1).

Bacterial α-diversity

Bacterial α-diversity, presented as the Shannon index, exhibited similar seasonal trends in all treatment combinations with the lowest diversity observed in June (Fig. 1a). Diversities were credibly higher in the treatment with manure when compared to the control treatment (HDI [-0.17; -0.01]). Plant residues did not change α-diversity compared to the control (HDI [-0.1; 0.02]) and showed a tendency for lower values than in manure fertilised plots (HDI [-0.01, 0,12]). Bacterial α-diversity was increased in plots fertilised with mineral N when compared to control plots without mineral N fertilisation (HDI [-0.13; -0.01]). Species evenness did not differ between organic fertilisation regimes. However, mineral N addition resulted in bacterial communities being slightly, but credibly, more even when compared to communities from plots without mineral fertiliser addition (HDI [-0.012; -0.003], Fig. 1b).

Bacterial β-diversity analysed by ordination

In a first analysis step, β-diversity was assessed with time and fertiliser regimes as predictors of community composition using partial CAP. The sampling date as well as fertilisation regimes proved to be significant determinants of community structure in a permutation test (date: F(3,53) =

3.65, $p < 0.001$; mineral: $F(1,53) = 5.21$, $p < 0.001$; organic: $F(2,53) = 4.16$, $p < 0.001$). The ordination plot indicated differentiation between the sampling campaigns in September and February from those in June and November, possibly due to a change from fallow soil to sugar beet cultivation (not shown). In a second step, the sampling date was partialled out in order to further focus the analysis on the influence of the fertilisation regime on bacterial community structure. This revealed a clear separation of plots amended with manure from those without organic amendments and amended with plant residues (Fig. 2). Both mineral N and organic amendments were significant factors of influence in the permutation test (mineral: $F(1,53) = 4.64$, $p < 0.001$; organic: $F(2,53) = 4.11$, $p < 0.001$). Variance was divided by CAP into 7.2 % constrained variance, 62.6 % conditional variance and 30.2 % unconstrained variance. From the taxa plot of the ordination the order Bacillales appears to be typical and indicative part of the bacterial community on the manure-amended plots as it appears close to the centroid of the manure plots.

Identification of main responders

A more detailed exploration of the taxa that are typical for the different variants of organic fertilisation was done using the BioMiCo algorithm. Prediction accuracy of affiliation to the three organic amendment groups in several runs of the analysis was consistently around 50 % and hence higher than the 33 % expected from random chance. Using the posterior probabilities for both OTU assemblages as well as OTUs within assemblages a subset of potential responders for the different fertiliser variants was chosen. Fig. 3 shows the absolute abundances of these responding bacterial groups as a heatmap. Visual interpretation of the graph reveals several species that appear to be more abundant in the plots fertilised with manure relative to the other treatments. The taxonomic identities of these OTUs harbour a striking proportion of members of the order Bacillales. To further support this observation, all taxonomic families within Bacilli were statistically compared between treatments (Fig. 4). The families Planococcaceae, Thermoactinomycetaceae and Turicibacteraceae as well as those Bacillales not assigned to any family proved to be credibly more abundant in the plots fertilised with manure in comparison to both the control and the plant residue treatment (cf. table 1).

Bacterial co-occurrence patterns

In order to compare the co-occurence pattern of bacterial communities, MetaMIS consensus networks were computed and general network structure was compared qualitatively between treatments. Predicted community compositions by the underlying model exhibited low Bray-Curtis dissimilarities of around 0.1, showing the accuracy of the approach. The resulting networks showed a similar structure for the plots to which no mineral fertilisation was applied (Fig. 5a-c). Two evident hub taxa, namely Kouleothrixaceae (phylum Chloroflexi) and Phormidiaceae (Cyanobacteria), connected by a strong positive edge originating from the former are present in each of the networks. Proportions of positive interactions are 21.0 %, 16.1 % and 16.4 % in control, manure and plant residues treatments, respectively, as indicated by red edges.

Network structure changed profoundly when mineral fertiliser had been added to the plots. Both the non-amended control plots and the plant residue treatments lacked the prominent positive network edge between Kouleothrixaceae and Phormidiaceae (Fig. 6a,c) and the portion of positive correlations dropped to 4.8 % and 3.2 %, respectively. However, from the plots fertilised with both mineral fertiliser and manure, the resulting network resembled more the ones without additional mineral fertilisation (Fig. 6b). The positive correlation highlighted above was present (represented by the red edge) as well as a higher overall portion of 22.2 % positive interactions. However, most of these positive interactions were originating from only one family, i.e. Chitinophagaceae, while in plots that did not receive mineral N they were spread out on several nodes in the network. The statistical evaluation of the network topology showed a credible increase in average path length for plots amended with organic fertilisers as compared to non-amended plots (HDI [-0.593; -0.115], Online Resource 1) indicating a more complex network structure. The type of organic amendment did not credibly influence average path length of co-occurrence networks (HDI [-0.152; 0.408]). Neither average degree nor the average clustering coefficient changed credibly due to fertilisation regimes (not shown).

Discussion

Bacilli as main responders to the amendment with organic fertilisers

General effects of fertilising practices on the soil microbiome are very comparable in studies from different parts of the world analysing different soil types [53-57]. Several authors state that mineral fertilisation practice is a major determinant of the bacterial community structure [54, 58-60]. Indeed also in this study the presence or absence of mineral fertilisation could be identified as a strong driver influencing the structure of the bacterial community. Possible explanations for this are the acidification of soil by NPK additions or the higher total nitrogen content [61, 62]. Another observation with empirical evidence from agriculture is the obvious mitigating effect of organic amendments like manure and straw on soil degradation by mineral fertilisers [55, 63]. Most commonly, higher bacterial diversity is found in soils amended with manure, but not straw, and this increase is then interpreted as one factor influencing soil fertility [60, 64, 65]. For the IOSDV trial, our study in fact demonstrates that manure exerts such an effect on bacterial diversity [66]. Accordingly, either of the organic fertilisers actually increases crop yield on these field sites. However, it should be noted that relating yield increases to bacterial community changes is problematic, because fertilisation affects both plant yield and the bacterial community. This is why the effects of the community on the crop yield cannot be easily distinguished from fertiliser effects and, hence, cannot be evaluated separately. To assess the effects of the bacterial community on yield, a future experimental approach would need to manipulate the bacterial community while keeping all other factors equal. However, this can likely be done only in a controlled pot experiment. In spite of the positive effects on overall diversity, abundance changes of soil-dwelling bacteria are usually relatively small and only connected to few species when compared to the more profound changes associated with NPK fertilisation [57]. Species that are often found to be typical for changes in fertiliser regimes, especially when contrasting pure mineral with organic amended soils, belong to the taxa Actinomycetales and Bacilli [57, 59, 64, 67]. In this study, recent bioinformatical tools were used to identify typical taxa within the different amendment treatments. The most evident changes were recorded in the class Bacilli, specifically in the order Bacillales,

where three families with credible changes due to manure addition to field sites were identified. It is hypothesised that these do not stem from the manure itself, as most species present in horse manure belong to the orders Clostridiales and Lactobacillales [68, 69]. Similarly, Chu et al. [63] described a specific 16S PCR band stemming from a *Bacillus sp.* species in manure-amended soil that was not present in the original amendment material. Bacteria from the Bacillus and Paenibacillus groups were found to play roles in denitrification, nitrogen fixation, cellulose, hemicellulose and pectin degradation as well as proteolysis [70-74]. What is more, most of the members are saprophytes [75, 76]. It seems therefore conclusive to find Bacillales species in the plots amended with manure that contains cellulose, hemicellulose as well as proteins. However, for the families found to be responders to the manure treatment in this study, knowledge is scarce. All of them, Planococcaceae, Thermoactinomycetaceae as well as Turicibacteraceae, seem to not be plant-associated as they were found to negatively correlate with living willow plant biomass [77]. This might hint to a similar, saprophytic life style as in other Bacillales. For the plant residue treatment in this experiment on the other side, no evidence was found for associated higher taxa. A possible explanation might be the chemical composition of the amendments, which does not drastically differ from e.g. rotting roots that are present in all plots after harvest. Microbes specialised on the decomposition of plant material dwell in all treatments and can therefore not be effectively used to distinguish the treatments in the present study. In addition to that it is conceivable that the treatments might prove to differ in their microbial community structure when taking other organisms, e.g. fungi and archaea, into account.

Bacterial co-occurrence

Using network statistics, general aspects of network topology can be compared and hence the influence of external factors on the interactive structure of bacterial networks can be estimated [78]. However, our results only consider the bacterial community, while other organisms, e.g. fungi and archaea, might play crucial roles in microbial interactions as well [79-83]. Nevertheless, we are confident that the immense abundance and well-known ecological importance of soil-dwelling bacteria enable us to depict a representative part of the community. In this study the hypothesis

that application of organic fertilisers will lead to a more complex co-occurrence pattern of the soil bacterial community was addressed. Indeed, it could be demonstrated that addition of organic amendments increases network complexity in soil. While earlier studies mainly focused on more general parameters like abundance, diversity or evenness [84, 85]; a change in co-occurrence as shown by this study directly enhances our understanding of the underlying mechanisms involved in the mitigation of detrimental NPK effects. Interestingly, an increased complexity of interactions caused by straw was also found. Without taking co-occurrence aspects into account, the influence of straw on the bacterial community might easily be underestimated. Indeed earlier studies found little effects of straw on the bacterial community, even though empirically positive effects on crop yield are known [12, 13, 66]. Another reason for studies to find only weak effects on the bacterial community might be short run times of the corresponding experiments as compared to the long-term experiment investigated in our study. Concerning the most evident taxa in the co-occurrence networks an exhaustive discussion is difficult due to the scarcity of information about those found in literature. However, as both Phormidiaceae (Cyanobacteria) and Kouleothrixaceae (Chloroflexi) belong to taxa that harbour photoautotrophic organisms, their relation might actually be limited to the very surface of the plots, e.g. in biological soil crusts [86]. These crusts could in turn be influenced by the increased plant cover in mineral-fertilised plots, thereby disrupting the relationship found in unfertilised plots with higher bare soil coverage. Other taxa with high connectivity in the networks described here are more likely to be present in deeper soil layers. The family Gaiellaceae with the only known member *Gaiella occulta* is able to reduce nitrate to nitrite and might therefore be part of the nitrogen cycle in soil [87]. Accordingly, it appears with higher connectivity in networks derived from the plots fertilised with mineral N, although this is reversed in the manure treatment. An evident difference to other treatments is the hub taxon Chitinophagaceae in the mineral-fertilised, manure amended plots, exhibiting exclusively positive interactions to other nodes. This might relate to the chitin and especially cellulose hydrolysing capability of *Chitinophagus* species [88, 89], presumably making metabolites of this pathway available to other organisms. Regarding the hypothesis that different soil amendments will affect co-occurrence networks differently, a qualitative comparison lets us conclude that the respective networks differ,

especially in mineral fertilised soils. This might be explained by the chemical differences of the organic material applied to the plots, whose decomposition involves other bacteria that will consequently have other interaction structures.

Conclusions

Former comparisons of soil amendments that did not take bacterial co-occurrence into account, might have underestimated the effect of straw on bacterial life in soil. Here, both manure and straw had a clear influence on the co-occurrence pattern of bacteria. While restricted to the bacterial community, these results clearly show the benefits and additional value of studying not only general parameters of microbial communities exposed to organic fertilisation, but also taking the interactive structure of the organisms into account. Future efforts should consider other organisms, expanded and denser time-series and experiments on smaller scale to enhance the resolution of the inferred interactions. Agricultural long-term experiments are an essential prerequisite for these investigations. While 16S sequencing analyses can guide to recognise key species in the centre of the observed changes, we need to find out about the specific functions these species are involved in. However, a possible step forward in understanding the effects of organic fertilisers on the community structure and function in soil requires the employment of novel methods. Future investigations should therefore comprise metagenomic and transcriptomic analyses of the communities as well as characterisations of single species with respect to their metabolism and life traits. Only then the ecology of the observed changes in interaction structures can be evaluated and explained.

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Tables

Table 1 Summary of statistical analysis of taxonomical families reacting to horse amendment with horse manure on field sites. The presented values are the most probably difference between the named treatment groups. Values in brackets give the lower and upper limit of the 95 % HDI. Credible differences between treatment groups are marked with *.

	comparison		
family	**manure vs. control**	**manure vs. plant residues**	**plant residues vs. control**
Planococcaceae	126 [109; 144]*	133 [114; 149]*	-6 [-15; 3]
Thermoactinomycetaceae	194 [166; 228]*	205 [177; 238]*	-11 [-25; 5]
Turicibacteraceae	15 [14; 16]*	15 [13; 17]*	0.3 [-0.2; 0.8]
not assigned	31 [25; 38]*	31 [24; 38]*	0.4 [-3.3; 4.0]

Figures

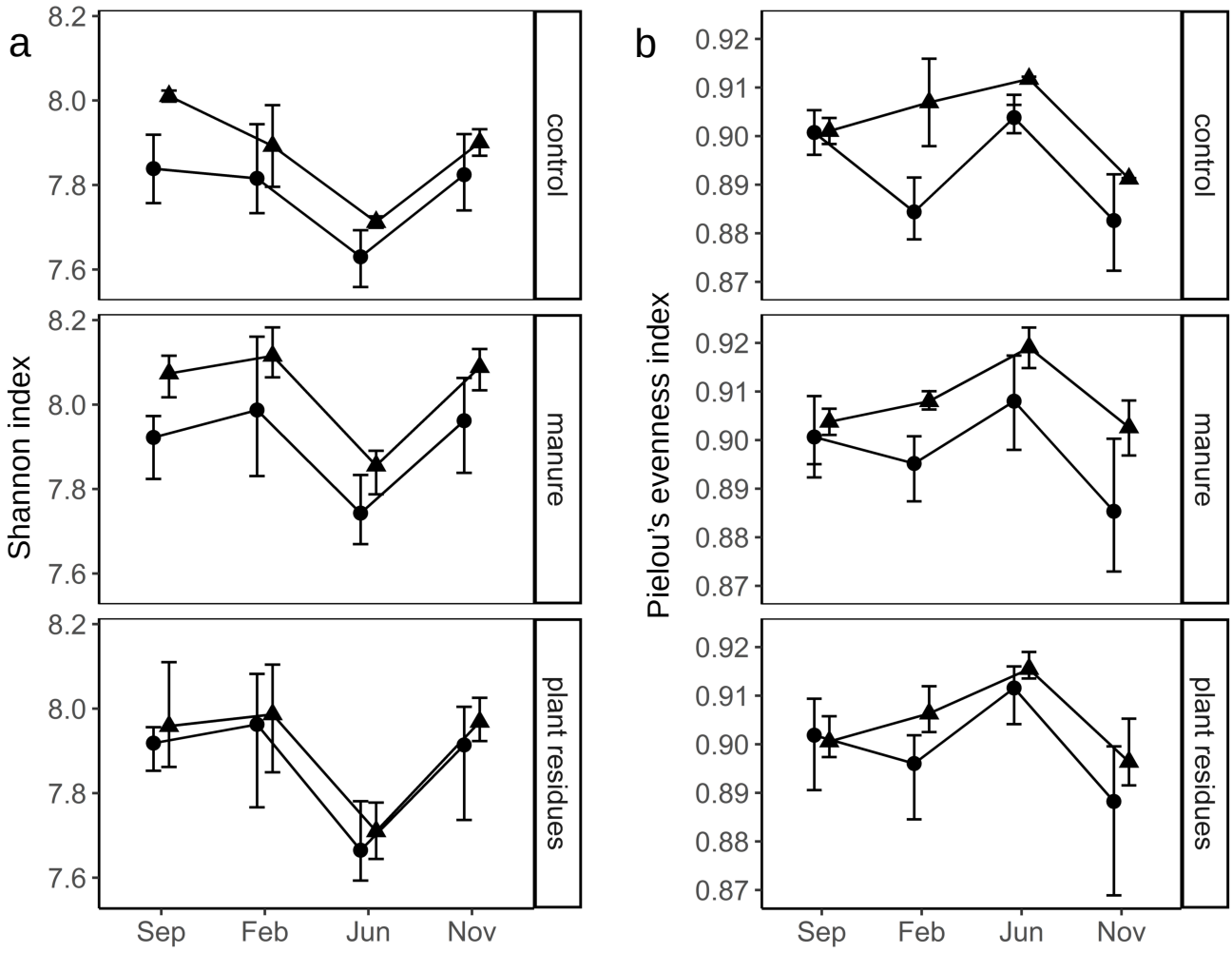

Fig 1 Alpha diversity (a) and species evenness (b). The values presented are mean values, errorbars show 95% confidence intervals. n = 3 (two for no organic, with mineral fertiliser). Circles represent plots without, triangles plots with addition of mineral N. For results of statistical analyses see text

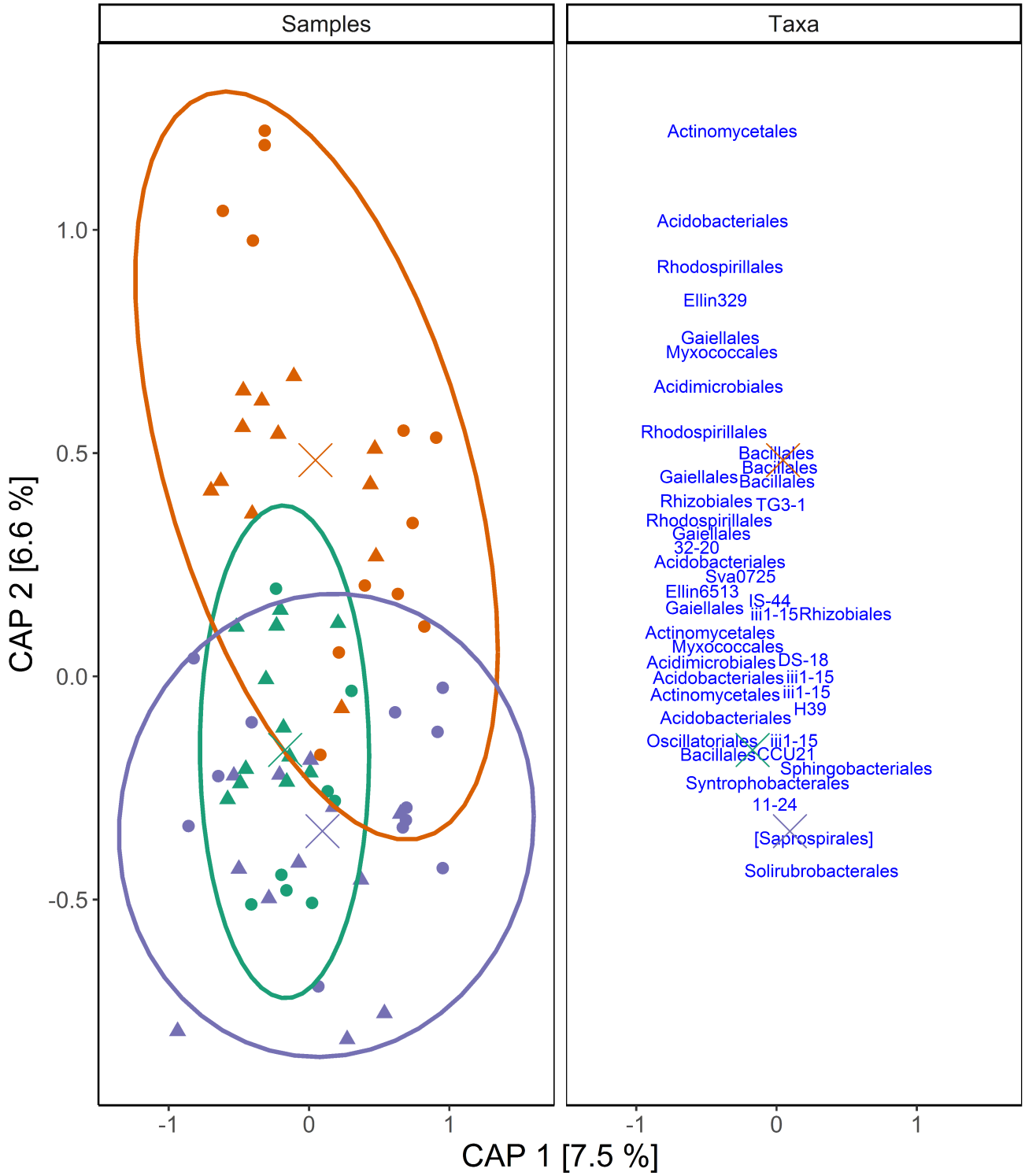

Fig 2 Partial constrained analysis of principal coordinates on generalised UniFrac distances. Samples scores are plotted on the left hand side, while taxa scores are plotted on the right hand side with reduced overlap to enhance readability. Centroids, i.e. the average cases per amendment group, are presented by "X" and can be used as a reference to find the most typical species in the taxa plot. Circles show samples from plots with, triangles from plots without mineral N fertilisation. Colours refer to soil amendment treatments with green, orange and purple representing control, manure and plant residue, respectively. For analysis details see text

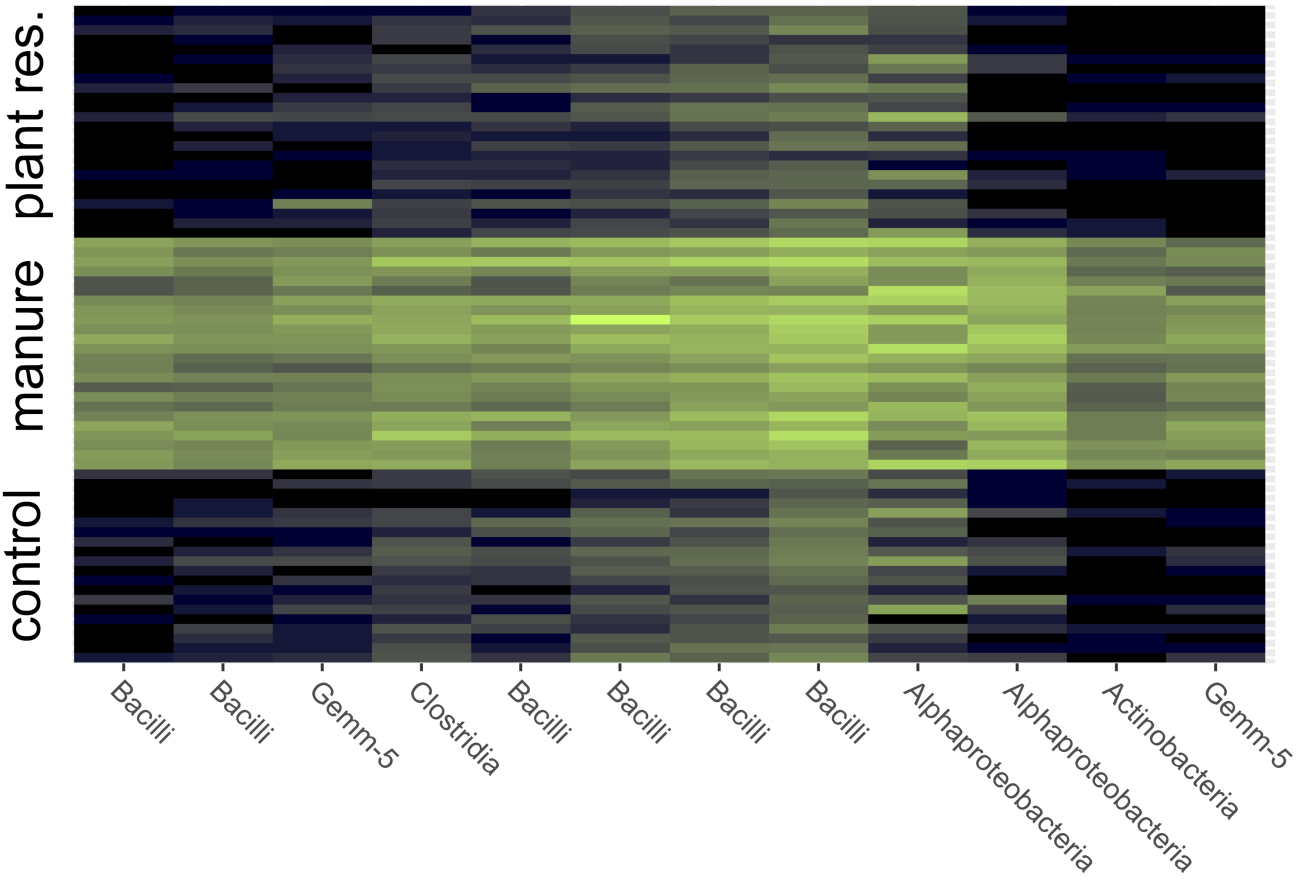

Fig 3 Heatmap of identified main responders to horse manure amendment. Shading is based on total abundance with black representing absence, and brighter shades of green representing an increasing abundance of the respective OTU in a sample. The x-axis shows the assigned taxonomic class of each OTU

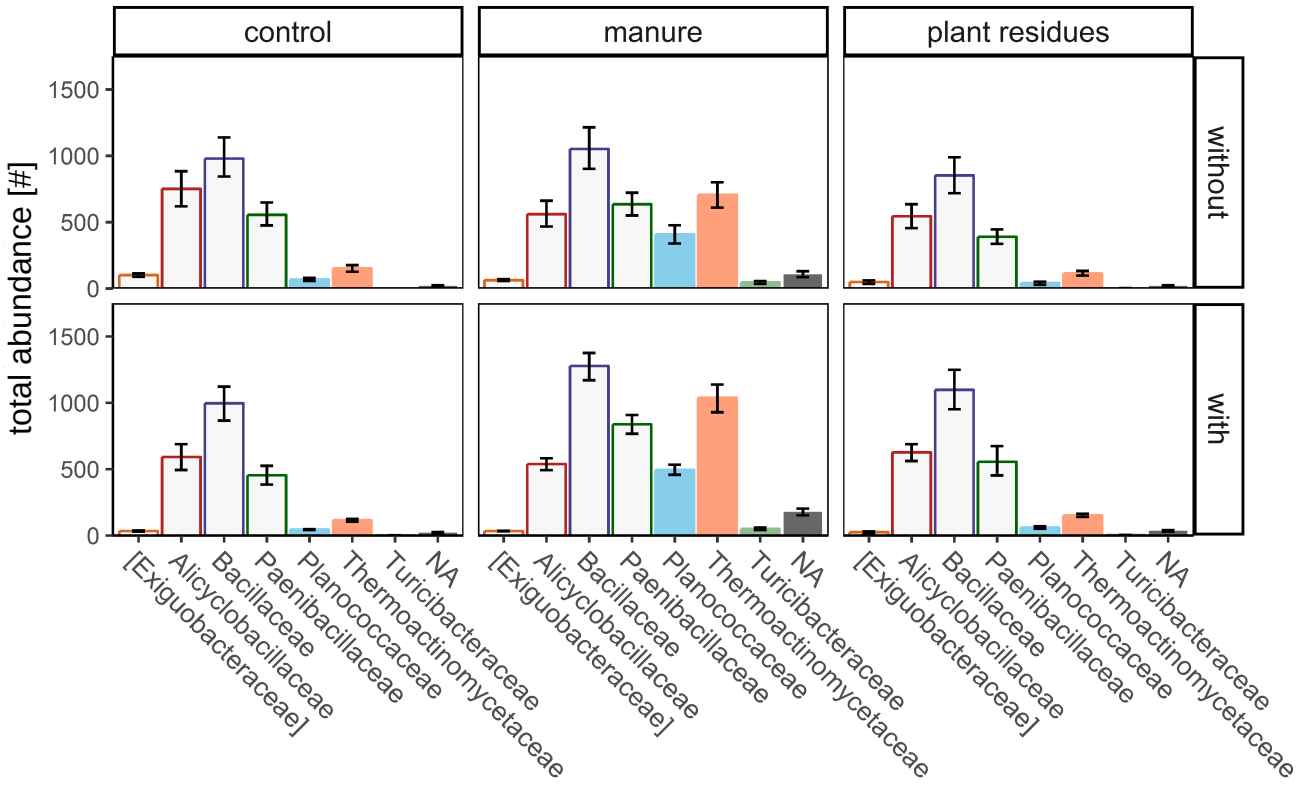

Fig 4 Total abundance of families within the order Bacillales. Presented values are total abundances averaged over the four sampling campaigns, error bars show 95% confidence intervals. Empty bars represent families not changing credibly between soil amendments, while filled bars represent families credibly enriched in the plots amended with horse manure

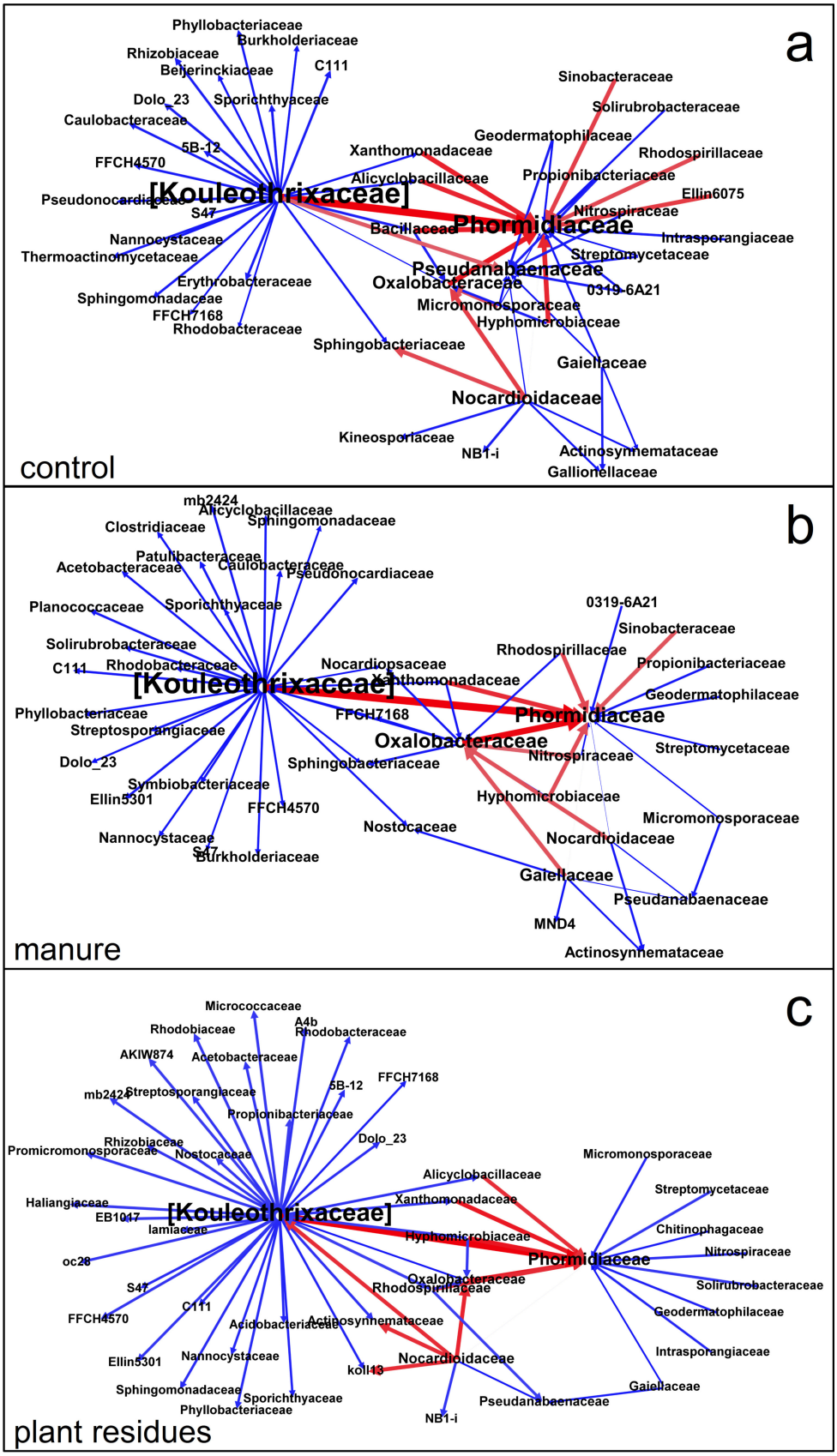

Fig 5 MetaMIS consensus networks of plots *without* addition of mineral N. Sub-plots represent the bacterial co-occurrence in control (a), manure (b) and plant residue (c) treatment. Red edges represent positive, blue edges represent negative correlation. Edge thickness corresponds to estimated interaction strength. The font size depicts the node degree, i.e. how many connections the corresponding family possesses in the network

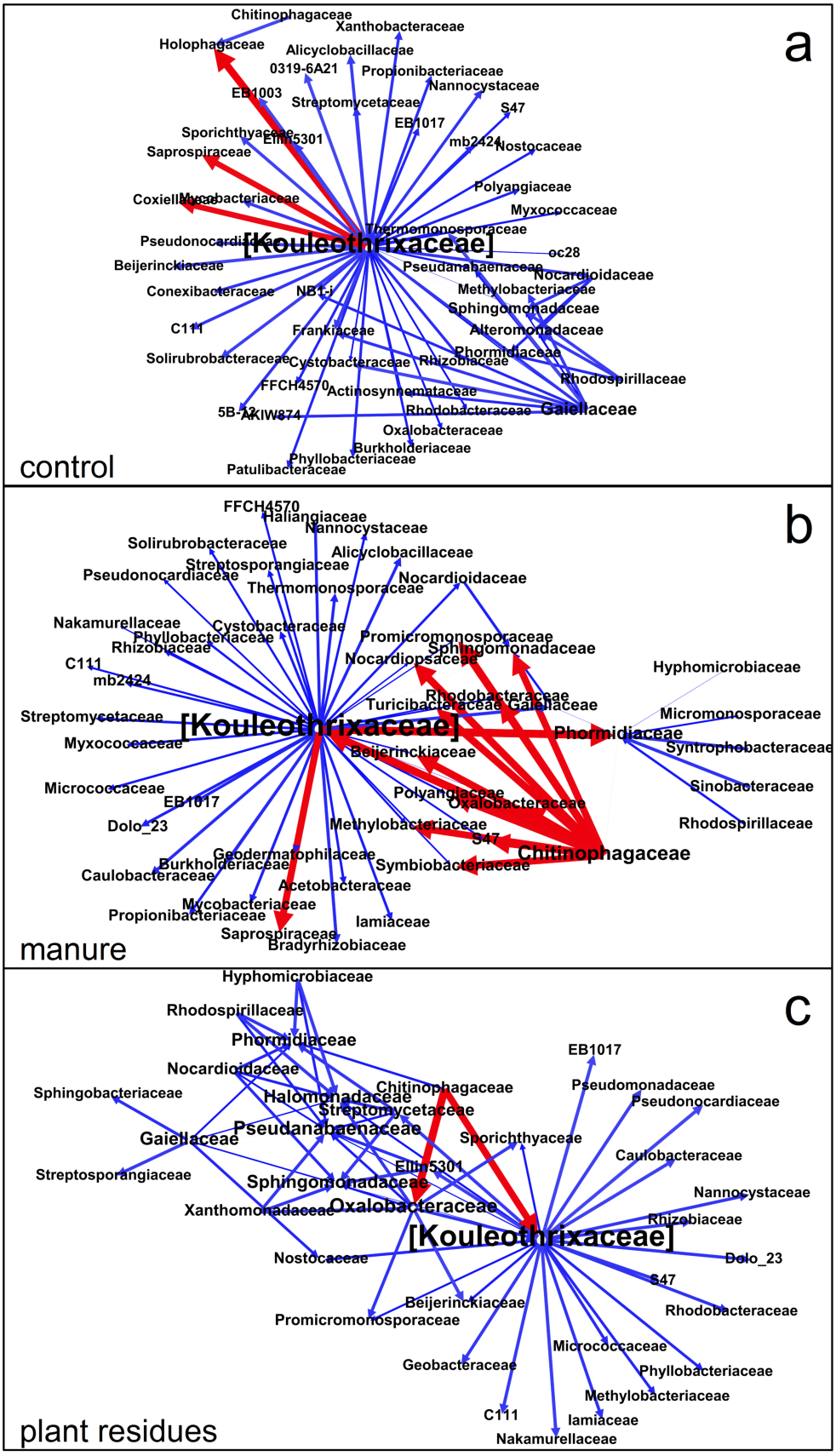

Fig 6 MetaMIS consensus networks of plots *with* addition of mineral N. Sub-plots represent the bacterial co-occurrence in control (a), manure (b) and plant residue (c) treatment. Red edges represent positive, blue edges represent negative correlation. Edge thickness corresponds to estimated interaction strength. The font size depicts the node degree, i.e. how many connections the corresponding family possesses in the network

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