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Soil microaggregates as habitat for microorganisms

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Es gibt keine Wunder. Nur wissenschaftlichen Fortschritt.

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Summary

Aggregation of soil particles is one of the most characteristic features of terrestrial soils and crucial for their functionality. Despite the high relevance of soil aggregates, only little is known about their genesis and functionality due to their complex and heterogeneous three-dimensional architecture. Furthermore, soil aggregates represent a major bacterial habitat, but so far neither the impact of aggregate structures on soil bacterial communities and their specific bacterial colonization are fully understood, nor the bacterial contribution to the formation of aggregate structures. Based on this, in this thesis I analyzed (I) whether different aggregate types have a formative influence on the soil bacterial community and (II) whether specific aggregate size classes provide preferential habitats for specific bacteria. In addition, I analyzed (III) whether bacteria are involved in the initial formation of aggregate structures and whether these structures in turn have an influence on bacterial life. Furthermore, I explored (IV) the possibilities of modern light microscopic systems and image processing tools in visualizing complex aggregate structures and their bacterial colonization.

To address effects on the bacterial community structure, I analyzed aggregates varying in size, clay content and, for microaggregates, localization in the soil as free or occluded within macroaggregates (I). This was done using size-fractionated soil samples from a clay catena. The results showed that the bacterial community was influenced by both clay content and aggregate size class. Whereby with increasing clay content an increased differentiation of the bacterial community was observed, especially in the smallest aggregate fraction. I used an indicator taxa analysis to test whether different aggregate size classes provide specific habitats for particular bacterial taxa (II). Here, I could demonstrate that especially in the smallest aggregate size fraction (<20 µm) differences in the general lifestyle of the indicator taxa were observable. Predominantly copiotrophic bacteria were identified to be enriched in the free aggregate fraction, whereas predominantly oligotrophic organisms were enriched in the occluded aggregate fraction, indicating that a further differentiation of the bacterial community occurs within this specific aggregate size fraction in dependence on the localization of the aggregates in the soil matrix. To investigate the initial aggregate genesis and the impact of these resulting aggregates on bacterial cells, I utilized montmorillonite and goethite as model minerals as well as different soil bacteria in the presence or absence of stress conditions in form of wetting and drying cycles (III). My analyses revealed that the presence of bacteria contributed to the formation of larger aggregate structures, whereby effects on aggregate formation were species-dependent (III). In addition, I demonstrated that the aggregate structures that were formed had a sheltering effect for the bacteria when applying wetting-drying cycles. Using a modern epifluorescence microscope, equipped with an ApoTome.2 and a color camera, I visualized a variety of microaggregates in real color along with their surficial bacterial colonization upon method establishment (IV). By applying specific staining approaches and modern image processing methods I was able to demonstrate the feasibility of visualizing complex soil microaggregates and observed a heterogeneous bacterial colonization on their surfaces.

In synthesis, my results thus show that in addition to size other aggregate properties or also the spatial localization of aggregate structures in the soil matrix, contribute to the diversification of bacterial communities in the soil. In this context, soil bacteria are not only beneficiaries of the available structures and given influencing factors, but also actively involved in the genesis of aggregate structures, with different bacterial species themselves exerting different influences. As a consequence, unique aggregate structures develop, which provide the foundation for a diverse soil bacterial community, demonstrating the fundamental importance of processes and effects at small spatial scales and their impact across larger and superordinate scales.

Zusammenfassung

Die Aggregation von Bodenpartikeln ist eines der charakteristischsten Kennzeichen terrestrischer Böden und ausschlaggebend für deren Funktionalität. Trotz der hohen Relevanz von Bodenaggregaten ist aufgrund ihrer komplexen und heterogenen dreidimensionalen Architektur nur wenig über ihre Entstehung und Funktionalität bekannt. Bodenaggregate stellen zudem ein bedeutendes bakterielles Habitat dar. Dennoch sind bisher weder die Auswirkungen von Aggregatstrukturen auf bakterielle Bodengemeinschaften und die spezifische bakterielle Besiedlung dieser, noch der bakterielle Beitrag zur Bildung von Aggregatstrukturen vollständig verstanden. Aufgrund dessen habe ich in dieser Arbeit analysiert, (I) ob verschiedene Aggregattypen einen prägenden Einfluss auf die bakterielle Bodengemeinschaft haben und (II) ob bestimmte Aggregatgrößenklassen bevorzugte Lebensräume für bestimmte Bakterien bereitstellen. Zudem habe ich analysiert, (III) ob Bakterien an der initialen Ausbildung von Aggregatstrukturen beteiligt sind und ob diese Strukturen ihrerseits Einfluss auf die Bakterien nehmen. Des Weiteren werde ich ergründen, (IV) welche Möglichkeiten modernste lichtmikroskopische Systeme sowie Bildauswertungssoftware bei der Visualisierung von komplexen Aggregatstrukturen und deren bakterieller Besiedlung bieten.

Um diesen Fragen nachzugehen, habe ich Aggregate mit unterschiedlicher Größe, unterschiedlichem Tongehalt und - im Falle von Mikroaggregaten - unterschiedlicher Lokalisierung im Boden in Form von freien oder in Makroaggregaten eingeschlossenen Aggregaten analysiert (I). Hierzu wurden größenfraktionierte Bodenproben aus einer Toposequenz mit Tongradient verwendet. Meine Ergebnisse zeigten, dass die Bakteriengemeinschaft sowohl vom Tongehalt als auch von der Aggregatgröße beeinflusst wurde, wobei mit steigendem Tongehalt eine zunehmende Differenzierung der Bakteriengemeinschaft zu beobachten war, insbesondere in der kleinsten Größenfraktion. Des Weiteren habe ich mithilfe einer Indikator-Taxa Analyse geprüft, ob unterschiedliche Aggregatgrößenklassen spezifische Habitate für Mikroorganismen bereitstellen (II). Hier konnte ich zeigen, dass insbesondere in der kleinsten Aggregatgrößenfraktion (<20 µm) Unterschiede in der generellen Lebensweise der Indikatortaxa zu beobachten waren. In der freien Aggregatfraktion wurden überwiegend copiotrophe Bakterien identifiziert, während in der okkludierten Aggregatfraktion vorwiegend oligotrophe Organismen angereichert waren, was darauf hindeutet, dass innerhalb dieser spezifischen Aggregatgrößenfraktion eine weitere Ausdifferenzierung der bakteriellen Gemeinschaft in Abhängigkeit von der Lokalisierung der Aggregate in der Bodenmatrix stattfindet. In einem weiteren Experiment habe ich die initiale Aggregatbildung und die Einflüsse dieser entstandenen Aggregate auf Bakterienzellen mithilfe von Montmorillonit and Goethit als Modellmineralien, sowie verschiedenen Bodenbakterien in Gegenwart bzw. Abwesenheit von Stress in Form von Nass- und Trockenzyklen, untersucht (III). Dabei ergaben meine Analysen, dass die Präsenz von Bakterien zur Bildung größerer Aggregatstrukturen beiträgt, wobei sich unterschiedliche Bakterienarten verschieden auf die Aggregatbildung auswirkten. Zudem konnte ich nachweisen, dass die entstandenen Aggregatstrukturen einen schützenden Effekt für die Bakterien hatten. Des Weiteren habe ich mithilfe eines modernen Epifluoreszenzmikroskops, welches zusätzlich mit einem ApoTome.2 und einer Farbkamera ausgestattet war, eine Vielzahl an Mikroaggregaten mitsamt ihrer bakteriellen Oberflächenbesiedlung visualisiert (IV). Somit konnte ich die Machbarkeit der Visualisierung komplexer Bodenaggregate in natürlichen Farben demonstrieren, wobei durch spezifische Färbung und die Anwendung moderner Bildauswertungsmethoden eine heterogene bakterielle Besiedlung auf der Oberfläche von ebenso heterogenen Aggregaten dargestellt wurde.

In der Zusammenschau zeigt sich somit, dass neben der Größe auch andere Aggregateigenschaften oder auch die räumliche Lokalisierung von Aggregatstrukturen in der Bodenmatrix zur Diversifizierung der bakteriellen Gemeinschaften im Boden beitragen. Dabei sind die Bodenbakterien nicht nur Nutznießer der verfügbaren Strukturen und gegebenen Einflussfaktoren, sondern aktiv an der Genese von Aggregatstrukturen beteiligt, wobei verschiedene Bakterien unterschiedlich Einfluss nehmen. Dies führt zur Ausbildung von einzigartigen Aggregatstrukturen, welche die Grundlage einer mannigfaltigen bakteriellen Besiedlung und somit auch einer hochdiversen bakteriellen Bodengemeinschaft bilden und verdeutlicht damit die grundlegende Relevanz, welche auch von Prozessen und Effekten in kleinen räumlichen Maßstäben und deren Auswirkungen auf größere und übergeordnete Skalen ausgeht.

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Al	Aluminum
C	Carbon
Ca	Calcium
CARD-FISH	Catalyzed reporter deposition fluorescence in situ hybridization
Cl	Chlorine
DAPI	4,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
EPMA	Electron probe micro analysis
EPS	Extracellular polysaccharides
Fe	Iron
FISH	Fluorescence in situ hybridization
IV	Indicator value
K	Potassium
N	Nitrogen
NHN	Normalhöhennull
OTU	Operational taxonomic unit
P	Phosphorus
PCR	Polymerase chain reaction
PI	Propidium Iodide
Si	Silicon
SSA	Specific surface area
UV	Ultraviolet

I
GENERAL INTRODUCTION

1 Rationale

The soil microbial community is one of the most diverse and species-rich on the planet (Quince et al., 2008) and, with approximately 2.6 x 10^{29} members (Whitman et al., 1998), an almost inconceivable quantity, one of the most extensive habitats for microorganisms. Estimates and studies on the number of microorganisms found in a single gram of soil range from 2000 to 8.3 million distinct prokaryotic species (Gans et al., 2005; Roesch et al., 2007; Schloss and Handelsman, 2006), which is not surprising considering that the surface area of this small amount of soil can exceed 800 m^2 (Pennell, 2016). Given these immense dimensions, it is astonishing that soil microbes cover only about 10^{-6} % of the surface, which, as it were by coincidence, corresponds to the same coverage of the land area of the earth that humankind populates (Young and Crawford, 2004). However, it is important to consider not only the absolute and average cover, which depends on a variation of a variety of factors such as the availability of nutrients and water or the pH, but also to be aware that even under the most auspicious conditions, the proportion of soil surface area inhabited is still less than 1% (Young and Crawford, 2004).

The fundamental structure that contributes to the formation of a multitude of different habitats and niches as well as the associated high bacterial diversity is the aggregate structure of terrestrial soils. Soil aggregates are built up according to a hierarchical concept, which starts with the smallest primary particles and leads to increasingly larger and ever complex soil structures through the accumulation, nesting and interaction of various constituents. The resulting different aggregate size classes are enclosed and traversed by a widely ramified network of pores of various sizes. This inter- and intra-connectivity, in turn, allows the various compounds to be supplied, connected, and potentially to be exchanged. In this context, Tisdall and Oades (1982) developed their widely acknowledged aggregate hierarchy model, which strives to provide a basic understanding of the structural framework of a large majority of terrestrial soils.

Taking all of these findings together, and considering in particular the heterogeneity of the soil system, it is easy to imagine that bacterial colonization of soil occurs in spatially separated habitats with sometimes limited mutual exchange. This spatial separation, in turn, allows the development of myriads of individual niches and habitats, which facilitates and promotes the establishment of highly individualized and specialized communities. Given the high number of individual microhabitats, it is therefore reasonable that soils have such high bacterial diversity.

In this context, the high bacterial abundance in soil systems also plays a major role in the functioning of biogeochemical cycles (Curtis and Sloan, 2005), which in turn influences the storage of carbon as well as, of course, the availability of nutrients. Thus, microorganisms and an understanding of how their communities are formed and influenced in one of their largest and most important habitats (Flemming and Wuertz, 2019) are highly relevant, as they are the backbone for sustaining and nourishing all living macroorganisms, including humans (Curtis, 2006). In addition, microorganisms influence a large part of the ecosystem services that each individual human, as well as our entire human society, relies on (often too much for granted) (Philippot et al., 2013; Vandenkoornhuyse et al., 2015).

Despite the high relevance and consciousness of the important role played by microorganisms in soils, there are still a large number of unresolved issues related to the functioning and development of microbial communities at the smallest scale and at the level of soil aggregates, not least due to the immense complexity of soil systems.
Therefore, the aim of this thesis is to explore the role of soil aggregates in the context of bacterial colonization and interaction and to raise awareness of the relevance of processes at the microscopic level and their impact on the macroscopic world.

2 State of the art

2.1 Soil aggregates as habitats for microorganisms

Terrestrial soils are the most diverse bacterial habitat (Thompson et al., 2017; Vos et al., 2013), a knowledge that is accompanied by a growing awareness of the significance of the microbial world to our lives (Thompson et al., 2017). However, this is also countered by an increasing awareness of how limited our understanding of the fundamental structures of soil still remains (Thompson et al., 2017; Totsche et al., 2018). In this context, one of the key components for the structure of soils in general and as a habitat for microorganisms in particular are soil aggregates, which will be described in more detail here.

2.1.1 The aggregate hierarchy model

For a major part of terrestrial soils, the existence of an aggregate structure as a result of pedogenesis can be observed. Thereby, aggregation as such can already be considered as one of the characteristic properties of soils (Churchman, 2010; Totsche et al., 2018). The concept of aggregate structure of soils or aggregated soil components, which are divided into macroaggregates (> 250 µm) and microaggregates (< 250 µm) dates back to Edwards and Bremner (1964, 1967), who studied the stability of soil and its structure when it was exposed to ultrasound. According to the authors' conception, macroaggregates comprise microaggregates, which differ in that the forces holding macroaggregates together are weaker than the forces sustaining the structure of microaggregates. Tisdall and Oades, in a series of publications from 1972 to 1978, further developed the proposition of Edwards and Bremner into the aggregate hierarchy model, which is still the basic framework of the theory of aggregated soils and identifies three physical categories: macroaggregates (2000-250 µm), microaggregates (250-20 µm), and primary particles (< 20 µm). Like the previous concept, this model is based on the resistance of soil structures to the impact of ultrasound. Thereby, the soil is revealed as a system of aggregated soil components, which in turn are composed of progressively smaller aggregated components, which in their collectivity as well as in their inter- and intra-connectivity form the structure of the soil.

In comparison to the previous model, Tisdall and Oades assumed that roots and soil fungi respectively their hyphae are responsible for the formation of the smaller aggregate structures into larger stable aggregate structures and are stabilized by them (Tisdall and Oades, 1982). The authors focused on the structural aspects of the aggregation process, according to

which different binding agents play a role in the stabilization and formation of aggregates at the various hierarchical levels of the aggregation process. These different binding agents are either persistent (e.g. humic substances or polymers), temporary (e.g. roots and hyphae but also bacteria or algae) or more volatile (e.g. polysaccharides, which are rapidly degraded in the presence of microorganisms). This general concept, or at least the presence of aggregate structures, can be observed in the majority of terrestrial soils, although it does not apply to all soils (Totsche et al., 2018). Considering the soil as a whole, the presence and abundance of micro- and macroaggregates has a formative influence on major soil properties, such as water content, organic carbon content, and niche availability as well as habitat variability. In this context, associations between aggregate structures and bacteria are typically the norm rather than the exception (Jocteur Monrozier et al., 1991; van Gestel et al., 1996).

In the research of aggregate structures and the initial conceptualization of the aggregate hierarchy model, the focus was mostly on cultivated soils and the analysis of the effects and influences of aggregate structures on agricultural production, with only a minor role and attention given to microorganisms. It was not until the mid-1980s that soil aggregates were also increasingly identified as hotspots of interactions between microorganisms and physiochemical as well as biogeochemical interactions and processes (Gupta and Germida, 2015). Based on this, it was hypothesized that aggregate structures provide an ideal habitat for microorganisms, which in turn play a crucial role in the transformation of dead biomass and organic carbon as well as essential nutrients through biological and biochemical activity (Elliott, 1986; Gupta and Germida, 2015). This hypothesis was further elaborated to the extent that bacteria and soil fungi colonize and shape different aspects of the aggregate structures. Along with this came the idea that different organisms prefer different features of habitats or even depend on them. This in turn could facilitate certain biological and biochemical processes, which are in some cases organism-specific, such as the mineralization of nutrients or the transformation of organic matter (Gupta and Germida, 2015).

Soil aggregates in general, and the associated idea of habitat function and the resulting biological heterogeneity in particular, are part of a comprehensive framework that has undergone constant development and revision since its original conception by Edwards and Bremner (1964). A detailed presentation of this whole framework and its developmental history is beyond the scope of this introduction, therefore for a detailed overview please refer to

the review by Six et al. (2004) and for the specific relevance of microaggregates to the review by Totsche et al. (2018).

Therefore, here and in the following, only the current findings related to the habitat function of soil aggregates for soil microorganisms, specifically for soil bacteria, but also to some extent for soil fungi are presented.

2.1.2 Soil aggregates functioning as microhabitats and the relevance of the spatial scale

In recent years, there has been an increasing awareness of the importance of the spatial context as well as the biogeography for the dynamics and biodiversity of soil bacterial communities (Gupta and Germida, 2015; Vos et al., 2013). This interest can be attributed in part to advances and opportunities as well as the potency of modern sequencing techniques. For example, Hanson et al. (2012) were able to demonstrate that approximately half of the variation in bacterial communities can be attributed to habitat structure, based on an evaluation of studies ranging from an intercontinental scale to a scale of a few meters. Similar observations have been made for macroorganisms such as plants and animals, for example, and are widely accepted for these (Cottenie, 2005; Vos et al., 2013). Although modern sequencing techniques and constant innovations in this field provide a variety of insights, in most cases it is the fact that the size of the studied samples by far exceeds the relevant habitat scale for a bacterium. Moreover, entire pools of aggregates are often analyzed at once, making it infeasible to draw conclusions about isolated aggregates.

Soil colonization occurs predominantly at the surface of soil particles and in pores filled or moistened by water (Chenu and Cosentino, 2011; Vos et al., 2013). Thereby, bacteria occur predominantly as single cells, but also in microcolonies or in small biofilms (O'Donnell et al., 2007; Or et al., 2007). Describing aggregates as habitats of soil bacteria, one has to consider that their surfaces as well as the pores traversing and surrounding them enable and induce the development of microhabitats, which in turn facilitate diverse bacterial communities (Rabbi et al., 2016; Ruamps et al., 2011). The emergence of these diverse microbial communities is in turn enabled by the fact that these microhabitats differ in their composition and availability of organic substrates, as well as in their physical and chemical properties (Bronick and Lal, 2005; Davinic et al., 2012; Gupta and Germida, 2015; Hattori, 1988; Kandeler et al., 2000; Lagomarsino et al., 2012; Six et al., 2004; Smith and Peay, 2014; Vos et al., 2013). Furthermore, compared to microaggregates, macroaggregates have been found to contain more

organic carbon and higher concentrations of, in chemical terms, not as complex (compared to microaggregates) organic matter (Davinic et al., 2012; Hofmockel et al., 2011). In addition to the diverse and fluctuating availability of nutrients, environmental conditions, like oxygen availability (Sexstone et al., 1985), can be assumed to vary likewise on a small scale, which also contributes to the formation of diverse niches and habitats. Other important abiotic factors influencing microorganisms at the smallest scale include the water content of the soil, the texture of the surface structure of the soil particles, gas exchange, or also the properties of the soil pores (Ding et al., 2013; Ebrahimi and Or, 2014; Kravchenko et al., 2013; O'Donnell et al., 2007; Wang et al., 2013). Thereby, soil pores can likewise be divided into different size classes, whereby the diameter of the pores in turn affects the interchange of nutrients and the accessibility for different organisms. In this regard, the impact of environmental conditions especially on microorganisms in the soil is to a large extent dependent on the respective size scale (O'Donnell et al., 2007), both in terms of observing but also in terms of surveying them, which should always be kept in mind.

The high degree of heterogeneity in soils also led Rillig et al. (2017) to propose soil aggregates as evolutionary incubators for soil microorganisms. The rationale for this concept is the diverse and inherently isolated nature of soil aggregates, which favors high-level and parallel evolution of individual bacterial communities. This theory would also provide another explanation for the high microbial diversity in soil systems (Jansson and Hofmockel, 2018). In addition, spatial separation and isolation could favor the formation of symbiotic dependencies in microbial communities that enable the completion of metabolic processes that cannot be fully carried out by individual organisms. For example, it is known that in the course of evolution, a few bacterial strains have lost the ability to synthesize the amino acids they need and therefore rely on neighboring cells to provide them (e.g. Mee et al., 2014). Furthermore, Zelezniak et al. (2015) were able to reconstruct that it is very likely that interactions between microorganisms occur in natural communities and that metabolic exchanges in form of mating incomplete or complementary metabolic pathways probably happens quite often. This could be advantageous under nutrient-poor conditions, as fewer resources are required when coupling metabolic processes. Furthermore, spatial separation not only facilitates the formation of synergistically and symbiotically connected communities, since these are better protected from external disturbances by their separation and can thus initially evolve undisturbed, but can also serve as protection against predators. This protection from predators results from the

observation that small pore diameters exclude larger predators. It has been found that organisms that inhabit pores with a diameter of <6 µm are mostly protected from predators of the microfauna, since the predators cannot access these small pores due to their own size (Elliott et al., 1980; Hattori, 1988; Heijnen and Veen, 1991; Kuikman et al., 1990). In addition to the protection from predators, smallest pore diameters also provide protection from various environmental factors such as desiccation or major fluctuations in pH (Bitton et al., 1976; Stotzky and Rem, 1966). However, there is also the risk that pore sizes that become too small no longer adequately ensure diffusion of nutrients, which in turn is a vital constraint. Fungal hyphae, for their part, are found predominantly in pores > 10 µm in diameter (Schack-Kirchner et al., 2000), implying that soil pores < 6 µm are likely to be reserved predominantly for bacteria and archaea. Consistent with this, a diverse selection of studies indicates that the highest bacterial activity is predominantly located in microaggregates (e.g. Neumann et al., 2013; Nie et al., 2014; Ranjard and Richaume, 2001), which in turn fits with the findings of Rabbi et al. (2016), who were able to show that smaller pores dominate in microaggregates. On the other hand the highest abundance of soil fungi can be classified in macroaggregates (Kandeler et al., 2000; Kihara et al., 2012). Regarding the influence of different aggregate size classes, a variety of studies show that this represents a determining factor for bacterial diversity (e.g. Davinic et al., 2012; Sessitsch et al., 2001; Trivedi et al., 2017), however, there are also studies that see no relationship between aggregate size and bacterial community structure (e.g. Blaud et al., 2014; Väisänen et al., 2005). Nevertheless, it is considered proven that bacteria preferentially form associations with organic matter and clay particles (Totsche et al., 2018), whereby clay minerals also influence bacterial community composition (Babin et al., 2013; Ding et al., 2013).

2.1.3 Involvement of bacteria in the generation of aggregate structures

In addition to the colonization of aggregate structures by bacteria, there are also indications that microorganisms are to some extent actively involved in the process of aggregate formation or at least influence this process (Watts et al., 2005), although this has not yet been finally clarified. For example, there is reason to believe that the diversity of bacteria and fungi prevalent in soils also influences the connectivity and porosity of aggregate structures during aggregate formation (Crawford et al., 2012). With regard to the involvement of bacteria and clay particles, Lünsdorf et al. (2000) observed that clay particles accumulated on bacterial cells and coated them. This formation of particle-bacteria associations could indicate that bacteria

are actively involved in aggregate formation and may even constitute the nucleus of aggregate formation in some cases. Furthermore, there are considerations that attribute a significant role to the presence of bacterial extracellular polysaccharides (EPS) in aggregate formation as well as in the stabilization of aggregates (e.g.; Kleber et al., 2015; Sandhya and Ali, 2015).

In summary, complex and diverse interactions exist between soil constituents and the microfauna colonizing them, beginning with the formation of aggregate structures and extending throughout the entire soil life cycle, with the consequence of forming a stabilizing aggregate soil architecture that also drives and is part of geochemical cycling (Crawford et al., 2012; Lynch, 1981; Lynch and Bragg, 1992).

2.2 The spatial scale in macro- and microecology

Microbial communities and their habitats in soils are characterized by a high spatial heterogeneity (Franklin and Mills, 2007). Thereby it is important to always be well aware of the scale of microorganisms (Vos et al., 2013) in comparison to the macroscopic world. For instance, looking at the specific surface area (SSA) of soil particles, it can be seen that it varies depending on the type and composition of the soil particles as well as on the analytical technique used, resulting in SSAs ranging from 60 to 800 m^2g^{-1} (Bin et al., 2007; Pennell, 2016, 2016). SSAs of different microaggregate fractions (≤ 250 µm) have been reported to be up to 20 m^2g^{-1} (Schweizer et al., 2019), though different aggregate components will again vary in the extent of their SSA (Guhra et al., 2019).

Assuming an average area of 1 $µm^2$ occupied by a single bacterium in soil, with a conservative presumption of 60 m^2g^{-1} and a number of 10^9 bacterial cells per gram of soil (Torsvik and Øvreås, 2002) there would be roughly 250 µm of space in between each individual cell given an even distribution of all cells. Young and Crawford (2004) made a similar assessment, assuming that typically <1% of the surface area of soil particles is colonized by bacteria. For a better illustration of the proportions to be considered, the following analogy can be made on a human scale: A bacterial cell in comparison to a microaggregate (1 µm : 250 µm) compares roughly to a human being in relation to the Großer Ölberg (460 m above NHN), the highest mountain in the Siebengebirge.

Considering the dimensions of a bacterium, it is not surprising that microorganisms interact with surface structures at the micro- to nanoscale (Hol and Dekker, 2014). This allows them to adapt to extremely miniscule landscape structures - in our predominantly macroecological

view - serving them as habitats.

With our macroecological perspective, which is shaped by our own human perspective and thus by our subjective perception, we perceive landscapes to be a composition and spatial expansion of elevations, valleys, plains, rivers, vegetation and organisms. In this context, landscapes, regardless of scale, can be defined as an arrangement of patterns that are interrelated to the inhabiting organisms and ecological processes being observed at any given time (Turner, 2005). Conceptualizing the environment surrounding us at our respective scales is described by the concepts of macroecology or landscape ecology. Nevertheless, it is scarce to observe in previous literature that the principles of landscape ecology, which are valid and recognized at a larger scale, are associated with microbial ecology (e.g. Battin et al., 2007). Thereby, landscape ecology deals with the same questions that are also highly relevant for soil microbial ecology, namely the reasons for and consequences of spatial heterogeneity. Here, heterogeneity is the measure of how distinct areas within a landscape are from each other and furthermore how this spatial structure interacts with the influencing and influenced organisms. Indeed, one of the main questions in landscape ecology is to determine the scale of the effect of individual and combined environmental parameters on colonizing organisms (Jackson and Fahrig, 2012, 2015; Miguet et al., 2016). By the scale of the effect, in this context, the sphere of impact of respective environmental parameters is meant, i.e., in which spatial extent the effect of an environmental parameter has a determining effect. Due to the small size scale, it is even more difficult to precisely determine the effect radius for soil systems than it is already the case in landscape ecology (Miguet et al., 2016). These difficulties arise in particular from the fact that with today's technical methods it is often not yet possible to resolve environmental parameters on a micrometer scale, and in those cases where this is possible, the survey is significantly more challenging than it is on a larger scale. In addition, due to the nested structure of soil components, it is extremely difficult to perform appropriate measurements at the respective points of interest.

The spatial distribution patterns of organisms result primarily from abiotic factors, such as climate and the shape/characteristics of the landscape. Resulting patches with characteristic features, resources and organisms are framed by boundaries that influence and control processes within the patches and thus regulate the movement of organisms across the landscape (Lovett et al., 2005; Reiners and Driese, 2004). For example, higher animals often move from one spot to another, which is necessary because of the uneven distribution of resources. In

this process, the heterogeneity of the landscape (from a macroecological perspective, for example: mountains, valleys or rivers; from a microecological point of view e.g.: micro- and macropores) has a reinforcing or inhibiting effect on the ability of organisms to move, which in turn affects the distribution and colonization of new habitats or even the invasion of an existing community. Thus, these processes can be found at both the macro-ecological level as well as the micro-ecological level. This relevance of environmental heterogeneity for non-host associated microorganisms is also highlighted in a review by Langenheder and Lindström (2019). Furthermore, Gupta et al. (2020) were able to show the influence of spatiotemporal parameters at the bacterial size scale on microbial communities utilizing a microfluidic platform to quantify spatiotemporal parameters.

On closer inspection and consideration of what constitutes landscapes, it can thus be said that microaggregates are microbial landscapes.

To this point, there is no full implementation of the concept of landscape ecology within microbial ecology, yet some studies have shown that microbial communities are influenced by spatial heterogeneity, even at small scales (e.g. Hol et al., 2016; Keymer et al., 2006; Salek et al., 2019). A distinctive feature of considering microbial communities is that, unlike the majority of macroecological communities, they are also capable of actively shaping and changing the environment around them through their activity (e.g. Bardgett and van der Putten, 2014; Schimel and Schaeffer, 2012). This, in turn, may impact how and which future generations of organisms colonize a then differently shaped environment.

2.2.1 Relating macro- and microecology

There are a large number of influential factors that shape and determine organisms and ecosystems, whereby large-scale factors are more easily recorded and thus also analyzed. In contrast, this is difficult at the microbial scale, where studies to date have been highly inconsistent with respect to the characteristics of ecosystems and microbial communities (Langenheder and Lindström, 2019). Nonetheless, it cannot be ignored that microorganisms interact with their immediate environment, and that in particular the composition of this environment has a particular influence on the heterogeneity of microbial communities, both spatially and temporally (Mony et al., 2020). If one aims at a direct comparison of ecological processes and their effects at the micro and macro scope, obviously there are some differences that can be identified. The first obvious difference is the magnitude of the organisms, which also affects the sphere of impact. However, the difference is put into perspective when one becomes aware

of the size relativities, as was done at the beginning of this section. Another not insignificant difference is the short generation time of microorganisms. If one takes the small size, the short generation time, and the related relevance of evolutionary processes together resulting in scales nested in space and time, the subsequent effects are in all likelihood considered to be more complex than in macroorganisms (Mony et al., 2020). Nevertheless, it is worthwhile to consider macroecological principles, such as those from landscape ecology, more closely and contemplate a translation of these to microbial ecology. It is therefore worth reflecting on whether microbial ecology has so far not paid sufficient attention to spatial, temporal as well as phylogenetic scales (Ladau and Eloe-Fadrosh, 2019).

McGill (2019) describes the aspiration of macroecology in identifying and ascribing generality to patterns of ecological units occurring. This aspiration can also be excellently applied to aggregated ecological units in soil, namely aggregate structures, although this requires a transformation of the scale at which these patterns are to be detected and explained. In soil, the high degree of spatial heterogeneity and the inhomogeneous distribution (patch structure) of resources result in microhabitats that allow parallel existence of organisms dependent on the same resources, enabling interspecific as well as intraspecific aggregation of microorganisms in aggregate structures (Etterma and Wardle, 2002). The formation of this aggregated structure inhabited by microbial communities can thereby be observed across different spatial scales (e.g. Bach et al., 2018; Bailey et al., 2013; Decaëns, 2010; O'Brien et al., 2016; Thakur et al., 2020). In addition, it should also be borne in mind that the environment as well as the sphere of impact of soil bacteria often corresponds to a scale where environmental parameters are usually not surveyed (Hendershot et al., 2017). Although there are a lot of open questions and knowledge gaps in the field of macroecology and the application of several of its principles in the field of soil ecology (Eisenhauer et al., 2017), it is worthwhile to take a closer look here and review what synergies there are or can be found. In line with this, there has recently been an increasing interest in linking macroecological and microecological concepts (e.g. ; Shade et al., 2018; Thakur et al., 2020), although it is a major challenge to integrate these concepts not only in terms of spatial as well as temporal dimensions, but also to establish an organized hierarchy of species, communities, and ecosystem (White et al., 2020). When applying macroecological approaches in the field of soil microbiology, it is also important to consider that the scale of most soil samples by far exceeds the sphere of impact of bacteria,

with the result of grouping together independent microbial communities that would be separated from each other in their natural habitat (microhabitats) and individual resource use and being without exchange with each other under undisturbed circumstances (Holden, 2011; Vos et al., 2013). This can subsequently affect or even bias observations of patterns and processes (Shoemaker et al., 2017), again emphasizing the relevance of respecting an appropriate monitoring scale.

2.3 Visualization of soil bacteria using light microscopy

The history of microscopy dates back to the early 15th century, when Johannes Kepler discovered that optical magnification could be achieved using convex lenses and oculars. From the middle of the 15th century, Antonie van Leeuwenhoek developed the first known microscopes, which made him the first person to observe and describe "animalcules", living organisms better known to us today as microorganisms. Nowadays, 6 centuries later, in the 21st century, microscopy has steadily evolved and, apart from light microscopy, has opened up many other exciting fields, embracing new techniques extending beyond visible light and enabling incredible magnifications and resolutions. Nevertheless, light microscopy is still widely used today and offers a wide range of possibilities. In the following, the current opportunities and applications of light microscopy in the visualization of soil bacteria in their natural habitat, the soil, will be elucidated.

To gain an understanding of soil bacterial ecology and communities at the level of individual colonies or even individual cells, it is important to accurately locate and contextualize the organisms in their surrounding environment at a high resolution appropriate to these bacteria. With a resolution limit of classical light microscopes around 0.2 - 0.3 µm (Abbe, 1873), this technique is sufficient to differentiate individual bacteria. When using fluorescence microscopes, the resolution limit can be even lower, depending on the wavelength used.

The microscopy of bacteria in pure culture respectively the observation of bacteria detached from their natural habitat is mostly uncomplicated. Even though it requires modifications of the light used for microscopy utilizing filters or staining of the cells, since bacteria are very difficult or even impossible to identify in classical light microscopy using environmental samples. One way to visualize bacteria under the microscope and to enable localization of bacteria in environmental samples is the use of fluorescence microscopy. Dyes that intercalate with DNA are usually used to visualize bacteria, such as DAPI (4,6-diamidino-2-phenylindole), a dye

that stains all bacterial cells in a sample and causing them to fluoresce when exposed to ultraviolet (UV) light. There is a wide range of dyes available for staining bacterial cells, some of which allow selective staining. One example of selective staining is the LIVE/DEAD BacLight staining kit, which allows living and dead cells to be distinguished by their respective fluorescence (Berney et al., 2007). Two dyes, SYTO 9, a green fluorescent and, Propidium Iodide (PI), a red fluorescent DNA dye, are used in this kit, which differ in their interaction with cell membranes. SYTO 9 stains all cells, whereas PI stains only cells with defective cell membranes, so that the SYTO 9 dye is superimposed by the red dye in dead cells, causing cells with intact cell membranes to fluoresce green and cells with defective cell membranes to fluoresce red.

One of the major challenges in visualizing bacteria in soil is the soil itself. Due to its heterogeneous structure and the predominant opacity, it is difficult to obtain an image of the bacterial colonization of soil particles using light microscopy. With the use of light microscopy, it is only possible to capture the surface of soil particles and the uppermost layer, which is penetrated by visible light. It is also challenging to capture the entire surface in a single image, as the focal plane is extremely shallow at high magnification. When applying fluorescence microscopy, another challenge in visualizing bacteria in soil is the occurrence of autofluorescence, and if fluorescent dyes are used, the non-specific binding of these (Li et al., 2004). This can lead to false-positive observations of bacteria when soil components either exhibit autofluorescence on their own, or were erroneously stained.

In order to investigate the spatial distribution of bacteria in the soil not only on the surface of soil particles, soil thin-sections are often utilized. The use of thin-sections has its origins in geology, having been used as early as 1964 by Jones and Griffiths to study the spatial distribution of microorganisms in soil aggregates. However, at that time, on the one hand, the technical possibilities for imaging bacteria using light microscopy were still very limited, and on the other hand, the techniques for dehydration and fixation of soil samples had not yet been sufficiently refined and optimized. Nowadays, these techniques are well elaborated and have already been used several times to detect the spatial distribution of bacteria in and on soil particles (Nunan et al., 2001; Nunan et al., 2003; Raynaud and Nunan, 2014).

The next major step in visualizing soil bacteria followed the development of fluorescent dyes, which for the first time opened up the possibility of quantifying soil bacteria in the soil itself (e.g. Tippkötter, 1990). This development now made it possible to differentiate bacteria dis-

tinctly from other soil components, however, the previously mentioned problem of autofluorescence as well as non-specific staining does not allow a decisive and definite quantification. The subsequent next significant step for microscopic analysis and evaluation of soil bacteria was the availability of digital cameras for capturing microscopic images, and the associated possibility of computer-assisted image analysis. Nunan et al. (2001; 2003) were thus able to generate fluorescence microscopic images of the in situ distribution of soil bacteria. These images provide, for the first time, an impression of the distribution of bacteria at a scale relevant to microorganisms. Not only individual bacteria or microcolonies and their immediate surroundings are imaged, but also neighboring organisms and their surrounding structures. Thus, for the first time, an impression of the distribution of bacteria in the soil system could be obtained. The persistent problem of non-specific binding of dyes to organic matter or other soil components was overcome a few years later with the help of new molecular biological advances. First, fluorescence in situ hybridization (FISH) was developed (DeLong et al., 1989) using fluorescently labeled oligonucleotide probes, allowing highly specific staining of bacteria. This method has also been used sporadically for soil samples (Bouvier and Del Giorgio, 2003; Eickhorst and Tippkötter, 2008a; Li et al., 2004) though the problem of nonspecific staining of organic matter often remained and increased autofluorescence was reported (Blagodatskaya and Kuzyakov, 2013; Hahn et al., 1993; Zarda et al., 1997). Through the advancement to the catalyzed reporter deposition (CARD)-FISH (Schönhuber et al., 1997) and continued optimization (Pernthaler et al., 2002), Eickhorst and Tippkötter (2008b) were finally successful in presenting a workflow for using CARD-FISH for soil samples. It was shown that this method is well suited for the specific detection of microorganisms in complex samples, such as soil samples. Thereby, the stained cells were well and clearly distinguishable against the background of the soil material, which was achieved by increased signal intensity and reduced autofluorescence. Nevertheless, this powerful method has found so far little further application in the visualization of soil bacteria using light microscopy. However, this may be due to the fact that this method is quite time-consuming and labor-intensive.

Nowadays, it is possible to conduct, automate and reproduce complex and highly accurate multidimensional imaging using motorized and digitized light microscopes. The high resolution and the high dynamic range of current digital cameras make it possible to capture high quality images under difficult lighting conditions. The high resolution of the images also makes it possible to use a lossless digital zoom in addition to the optical magnification, which enables

further magnification. Furthermore, the processing power of modern computer systems makes it possible to evaluate and analyze the generated images. Moreover, with the help of technical enhancements, it is possible to generate optical thin sections of fluorescent samples and thus generate 3D visualizations from 2D images, which allow a better impression and understanding of the spatial dimensions and nature of the bacterial environment. Using the potential of modern light microscopy in conjunction with older and proven methods will allow exciting and diverse new possibilities and insights to be explored and developed.

3 Objectives

Soils provide the foundation for almost all terrestrial life and at the same time harbor the greatest diversity of bacterial life. Despite this prominent role of terrestrial soils, the genesis and functioning of the smallest functional soil structures/building blocks, the soil aggregates and their building units, is still relatively obscure. The present study investigates the mutual influence and effects of microorganisms on soil aggregates. On the one hand, the influence of existing aggregate structures and their varying characteristics on bacterial communities have been analyzed. On the other hand, it was studied whether bacteria themselves play an active role in the formation of aggregate structures.

In the investigation of all questions, special attention will be paid to the microscopic dimensions considered and whether microscopic findings are also reflected in macroscopic dimensions, and vice versa.

The following questions will be explored in detail:

I. **Do varying aggregate properties such as size class, localization in the soil structure as well as variable clay content influence bacterial community composition and shape, allowing the identification and differentiation of specific communities?**
   The majority of all terrestrial soils consists of aggregated units of different soil components, which in turn results in a highly heterogeneous and complex system. This circumstance allows for a potentially wide variety of habitats and microniches, which favor the formation of different bacterial communities. Furthermore, it is well known that clay minerals are closely associated with soil bacteria, often resulting in an increased occurrence of bacteria in the size fraction of clay minerals or in association with them. Therefore, in order to investigate the influence of different aggregate classes, I will examine a wide range of different size classes as well as distinguish their localization in the soil structure as occluded in larger aggregate structures versus free-occurring aggregates. In addition, I will systematically investigate the influence of a variable clay content on the configuration of the bacterial community of different aggregate size classes.

II. **Do certain size categories of soil aggregates provide particular habitats which in turn favor specific bacterial taxa? If this is the case, can particular characteristics of these size categories be identified explaining this?**

A ubiquitous distribution of bacteria in the soil system is oftentimes presumed. Can a specific distribution of certain taxa be observed when looking at different aggregate structures at a fine scale?

III. **Do bacteria have a general influence on the initial phase of aggregate formation and do sundry bacterial taxa influence their environment in different ways? Furthermore, do the aggregate structures that form for their part also have an influence on the bacteria that colonize them?**

The processes involved in the initial formation of aggregate structures are as yet largely unknown. However, some observations and characteristics of bacteria indicate that they could be involved in this process. For instance, it is known that bacteria not only have the ability to adapt to their environment, but also to actively shape it to a certain extent. With the help of a model experiment with different soil building units as well as diverse bacterial strains and the simulation of natural wet and dry periods as abiotic factors, I will investigate the influence of bacteria on the initial aggregate formation and also analyze the reciprocal influence of aggregate structures on the bacteria themselves.

IV. **What possibilities do modern light microscopic methods open up in the spatial visualization of complex soil structures and their bacterial colonization?**

Due to the complexity and heterogeneity, light microscopic analysis of soil particles is a challenging task. Using state-of-the-art light microscopic techniques, I aimed to image bacteria in their complex natural habitat, the microaggregates. Thereby, the unaltered natural preservation of the soil particles during sample preparation as well as an impartation of the spatial aspect should be aspired.

II

CLAY CONTENT MODULATES DIFFERENCES IN BACTERIAL COMMUNITY STRUCTURE IN SOIL AGGREGATES OF DIFFERENT SIZE

Modified on the basis of

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1 Introduction

Soil is characterized by a highly complex and heterogeneous modular structure, which offers a tremendous degree of spatial and temporal heterogeneity and thus facilitates the formation of diverse and specialized microbial communities (Tecon and Or, 2017). Considering that the surface area of one gram of soil can exceed 800 m^2 (Pennell, 2016) and that usually less than 1% of the soil surface is populated by bacteria (Young and Crawford, 2004), one can assume that bacteria in soil are located in diverse spatially separated habitats. These provide highly variable niches due to the soil's inherent heterogeneity, which implies that the entirety of bacterially populated habitats can spawn communities with diverse specialized individuals.

Soil aggregation, a process that occurs in most soils as a consequence of pedogenesis, affects soil structure and contributes to the formation of heterogenous habitats for microorganisms (Totsche et al., 2018; Wilpiszeski et al., 2019). Tisdall and Oades (1982) describe the soil matrix as hierarchical system compiled of aggregates of different size classes with macroaggregates as soil structures >250 µm in comparison to the mechanically more stable microaggregates with a size <250 µm. Furthermore, small microaggregates with a size <20 µm are seen as structural building blocks of larger soil aggregates (Totsche et al., 2018). Macro- and microaggregates are composite units that form in response to abiotic processes and biotic activities introducing organic carbon into the soil. Aggregate formation is stimulated by processes exerting mechanical forces, e.g. wetting/drying, freeze/thaw or agricultural management practices such as tillage (Christensen, 2001). Differences in the bacterial colonization have been reported between macro- and microaggregates or specific size-fractions within these aggregate types (Bach et al., 2018; Bailey et al., 2013; Davinic et al., 2012; Sessitsch et al., 2001; Trivedi et al., 2017) and/or between microaggregates and the smaller sized (silt and clay) fraction (Davinic et al., 2012; Fox et al., 2018; Trivedi et al., 2015), thus confirming the relevance of aggregation for habitat diversification. However, it remains currently unclear whether differences are indeed best explained by these specific and commonly defined aggregate size entities, or whether differences are more specifically or additionally related to other aggregate size transitions, as most studies focus on a few different aggregate size fractions.

Soil aggregates differ not only in size, but also in other physical and chemical properties that may modulate microbial colonization, such as clay content. Clay minerals are known to affect microaggregate formation (Krause et al., 2018; Schweizer et al., 2019), and experiments with artificial soil have shown that clay minerals have an influence on the composition of bacterial

communities (Babin et al., 2013; Ding et al., 2013). However, no systematic investigation of the role of aggregation for microbial communities in dependence on soil clay content has been performed yet, especially not with aggregates from natural soils. Another feature that may lead to differences in aggregate properties and possibly linked to it the formation of different microbial habitats is the localization of microaggregates in the soil matrix either within stable macroaggregates or as free units in the soil matrix. It is well-known that microaggregates can be found as occluded components within macroaggregates, where they are probably more easily disconnected from water and nutrient flow, or as free microaggregates in the soil matrix (Angers et al., 1997; Oades, 1984; Six et al., 2002).

To study the role of different aggregate characteristics on bacterial communities in soil, we analyzed soil aggregates subdivided into ten different size fractions ranging from 8000 to <20 µm, thus covering several different size classes of macroaggregates and microaggregates, respectively. This allowed us to assess the importance of aggregate size in detail and to identify potential size thresholds that are linked to changes in bacterial colonization, based on the hypothesis that aggregates of different sizes support different types of habitats, resulting in differences in bacterial community composition. Moreover, we evaluated the relevance of aggregation for bacterial communities in dependence on soil clay content, which we hypothesized to modulate aggregate properties and thus the bacterial colonization of aggregates. We further differentiated between free and occluded microaggregates, as we hypothesized that the specific localization of microaggregates within the soil matrix contributes to variation in bacterial colonization. We therefore separated free microaggregates, which were released after a wet-sieving procedure, and occluded microaggregates, which originated from stable macroaggregates and were obtained upon macroaggregate disruption. We performed our study with Cambisol soil samples from a well-studied arable plot (e.g. Schröder et al., 2002), which shows spatial variation in clay content (Heggemann et al., 2017). As all samples were taken from the same plot, they underwent the same agricultural management and climate history. Upon soil fractionation, we characterized the bacterial community composition based on 16S rRNA gene sequence analysis.

2 Material and methods

2.1 Sampling site

Soil samples were collected from an agricultural plot at the research station Scheyern north of Munich in Germany (48° 29’ 36” N, 11° 26’ 15” E), which shows spatial variation in clay

content (Schröder et al., 2002). Soil samples were taken in late October 2015 in a field fresh state after the removal of the upper Ap layer (first 4 to 5 cm) utilizing a core cutter (diameter: 16 cm, length: 15 cm, volume: ~3000 cm^2), sieved to <8 mm and stored at 4 °C until further processing. Four replicate samples were analyzed per clay content level. The mean clay content of the replicate samples was 19, 22, 24, 32 and 34%, as determined by Krause et al. (2018). A more detailed description of the field sampling procedure is also given in Krause et al. (2018).

2.2 Wet fractionation

The field-moist soil samples were fractionated by wet sieving as described by Krause et al. (2018). The gained size fractions were: 8-2.8 mm, 2.8-2 mm, 2000-500 µm and 500-250 µm for macroaggregates, as well as 250-53 µm and 53-20 µm for microaggregates and <20 µm for aggregate building units. The latter corresponds to the silt and clay fraction in other studies. In the figures and tables of this study, the aggregate size fractions are denoted based on their upper size limit. It should be noted that all fractions contained also primary mineral particles of the respective size (quantified by Krause et al. 2018). All fractions <250 µm obtained after wet sieving were defined as free microaggregates, while the size fractions >250 µm were pooled, treated by ultrasonication (60 J ml^{-1}) and again subjected to wet sieving to gain occluded microaggregates and stable macroaggregates of different size classes that resisted the ultrasound. Different to the protocol of Krause et al. (2018), the <20 µm fraction was not collected by pressure filtration but by centrifugation of an aliquot of the fractionation water in this study. Sequential centrifugation steps were performed at 4°C to concentrate the particles, starting with a volume of 8 × 800 ml, which was centrifuged for 15 minutes at 4600 g using a swing-out rotor (Multifuge 4KR, Heraeus, Hanau, Germany). The supernatant was carefully discarded, the pellets were resuspended, combined and centrifuged again applying the same conditions. Subsequently, the pellets were pooled again, transferred to 50-ml tubes and centrifuged for 15 minutes at 4250 g (Allegra X-30R, Beckman Coulter, California, USA) in a swing-out rotor. Finally, resuspended pellets were pooled in 15-ml tubes, centrifuged as before and finally collected at 10,000 g for 15 minutes using a fixed angle rotor. All samples were lyophilized after collection and stored at -20 °C until further processing.

2.3 DNA extraction and amplicon sequencing

DNA was extracted from the different soil fractions using the NucleoSpin® Soil Kit (Macherey Nagel, Düren, Germany) following the manufacturer's instructions with the following adjustments: 0.3 g of soil were resuspended in SL1 buffer and the provided enhancer, microbial cell lysis was performed with a FastPrep®-96 instrument (MP Biomedicals, Santa Ana, California, USA) at 1200 rpm for 1 min, and the extracted DNA was finally resuspended in 30 µl of PCR-grade water. We obtained between 0.05 and 4.7 µg DNA per g dry soil (Suppl. Tab. II-1).

Amplification of the 16S rRNA genes was done using the barcoded primer set 515F-806R (Frindte et al., 2019), which targets the V4-V5 region of the gene. A two-step PCR procedure was applied to generate PCR products for amplicon sequencing. In the first step, primers without barcodes were used to amplify the genomic target region in 27 cycles in triplicate assays per sample using 1 µl DNA extract in a 10-µl assay. In the second step, the triplicate PCR products were pooled and served as template to amplify the target gene with 5 cycles using sample specific barcode primers in 50-µl PCR assays. The exact PCR conditions and all following steps including the quantification of PCR products, pooling, purification and the amplicon sequencing on an Illumina HiSeq instrument (2 x 250 bp) were performed as described by Maarastawi et al. (2018). Sequence files were submitted to the European Nucleotide Archive (ENA) database and are available under the project accession number PRJEB36920.

2.4 Sequence analysis

The sequences of forward and reverse reads were merged with USEARCH (Edgar and Flyvbjerg, 2015), afterwards reverse primers and sequences <200 bp were removed using *Cutadapt* (Martin, 2011). Sequences identified as reverse complementary were turned with fastx_reverse_complement (http://hannonlab.cshl.edu/fastx_toolkit). Demultiplexing was done with a customized perl script and barcoded forward primer sequences were removed. Further quality filtering was done using USEARCH v9 (Edgar, 2013) applying a q of >3 as recommended by Bokulich et al. (2013). A dereplication step was performed (Usearch fastx_unique) and sequences were clustered into operational taxonomic units (OTUs) using 97% sequence similarity as cut-off. A biom file was constructed using the usearch -usearch_global function. Taxonomy was predicted using the Usearch sintax command and the reduced RDP dataset (rdp_16s_v16.udb, https://drive5.com/usearch/manual/sintax_downloads.html). The OTU table was filtered to exclude very rare OTUs that occurred less than three

times across all samples and to exclude chloroplasts. The resulting biom file was used for further data analysis in R.

2.5 Statistical analysis of community compositional data

Data analyses and statistical evaluations were performed using R and the R packages Vegan (Oksanen et al., 2019), Phyloseq (McMurdie and Holmes, 2013), Microbiome (Lahti et al., 2017) and PMCMRplus (https://CRAN.R-project.org/package=PMCMRplus). For statistical analysis of alpha-diversity based on the Chao1 index and Pielou's evenness a rarefied OTU table based on 4732 reads per sample was used. Significant differences in alpha diversity between groups of samples were evaluated based on a non-parametric Kruskal-Wallis test, as a Shapiro-Wilk normality test revealed non-normal data distribution. When necessary, a Tukey-Kramer-Nemenyi post-hoc test was performed afterwards. Post-hoc tests were considered significant with a threshold of $p < 0.05$. Bacterial community composition was compared between samples using two approaches. On the one hand, Bray-Curtis distance metrics were calculated and non-metric multidimensional scaling (NMDS) plots were generated. On the other hand, a more recently proposed method using a centered log-ratio (CLR) transformation of the data followed by a Principal Component Analysis (PCA) as recommended by Gloor et al. (2017) was used, which is based on Euclidean distances to describe differences between samples. The latter approach has been reported to better take into account the dependency of the data, considering that relative abundance data are used. Statistical evaluation of differences in the bacterial community composition between groups of samples was performed by applying an analysis of similarity (ANOSIM) with 999 permutations. To further analyze the influence of different grouping variables on the variation in bacterial community composition between samples, a variation partitioning analysis was performed based on a Hellinger transformed version of the OTU table. Lastly, we performed an indicator taxa analysis (Dufrêne and Legendre, 1997) using the rarefied OTU table and calculated indicator values (IV) to detect characteristic OTUs in different aggregate size fractions using the R package indicspecies (Cáceres and Legendre, 2009). The p-values obtained by the multipatt function in the indicspecies package were corrected by the Benjamini and Hochberg p-value adjustment procedure to account for multiple comparisons. In the indicator taxa analysis an indicator index is calculated based on the concept of specificity (highest if a species only occurs in the target group) and fidelity (highest if the species occurs in all replicates of the target group). The closer the IV is to 1, the more characteristic is the species for the factor considered in the analysis. Three

levels of significance were defined in this study as indicated by asterisks (not significant, p >0.05, no symbol; significant, p ≤0.05, *; very significant, p ≤0.01, **; highly significant, p ≤0.001, ***).

3 Results

3.1 Changes in bacterial diversity due to aggregate size fraction and clay content

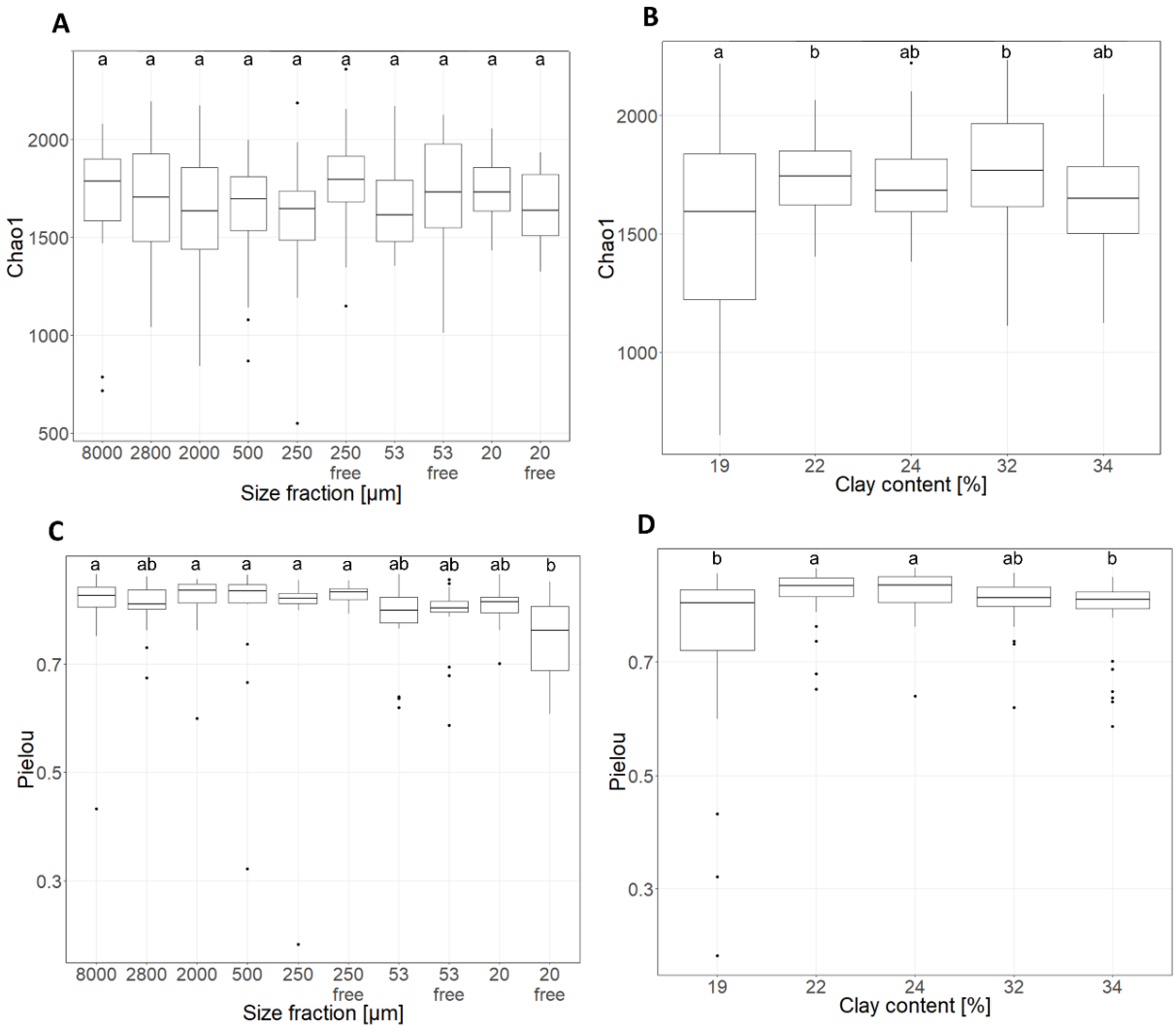

Fig. II-1: Box plots showing the influence of aggregate size fraction (A, C) and clay content (B, D) on bacterial diversity (A, B) and evenness (C, D), which were estimated based on the Chao1 and Pielou index, respectively. The box displays the 25th and 75th percentile whereas the line within the box marks the median and dots display outliers. Letters above the box plots indicate significant differences between groups of samples.

Bacterial community compositional analysis based on 16S rRNA gene amplicon sequencing was successful for 191 out of 200 samples. These were obtained from soils of five different clay contents, separated into ten different fractions representing macroaggregates, free and

occluded microaggregates, as well as aggregate building units (i.e. <20 µm). Alpha diversity was analyzed by comparing the estimated total species richness based on the Chao1 index and the evenness according to Pielou's evenness index (Fig.II-1). Statistical evaluation of differences in estimated species richness showed no significant differences in dependence on size fraction ($p = 0.106$). Slight differences were observed between samples of different clay content ($p = 0.005$), whereby the diversity in the samples from soil with 19% clay was slightly lower than in those with 22% and 32% clay.

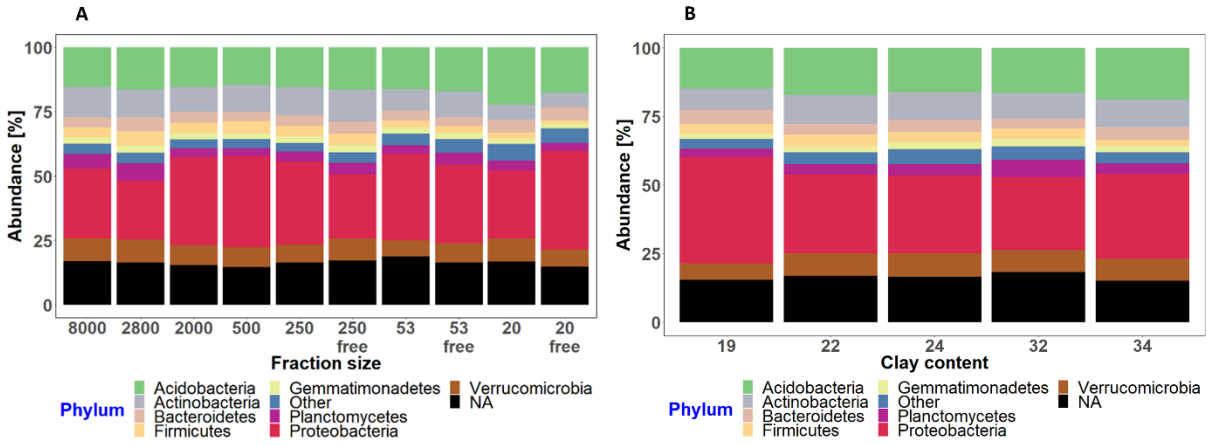

Fig. II-2: Relative abundance of bacterial phyla detected in the different aggregate size fractions (A) and from soils of different clay content (B). Phyla with a mean relative abundance < 2% are grouped as other. NAs represent sequence reads that could not be assigned to a specific phylum.

Pielou's evenness index was determined to evaluate whether the detected species occur with rather equal relative abundances (even; values close to 1), or whether the bacterial community is dominated by a few taxa with high relative abundance (values towards 0). The different size fractions showed only slight differences in evenness ($p \leq 0.001$). Post-hoc tests revealed that especially the fraction of free microaggregates <20 µm had a less even community composition than the aggregates of the 8000, 2000, 500, 250 µm occluded and 250 µm free fraction (Fig.II-1 C). Likewise, only slight differences were seen in dependence on the soil clay content ($p \leq 0.001$). A significantly lower evenness was observed in samples of the lowest and highest clay content (19% and 34%) compared to those samples with intermediate clay contents (22% and 24%) (Fig.II-1 D). The rather even distribution of the bacterial taxa can also be deduced when looking at the relative abundance of identified bacterial orders (Suppl. Fig. VIII-1), but is not well preserved up to phylum level (Fig.II-2).

3.2 Impact of clay content and aggregate size fraction on bacterial community composition

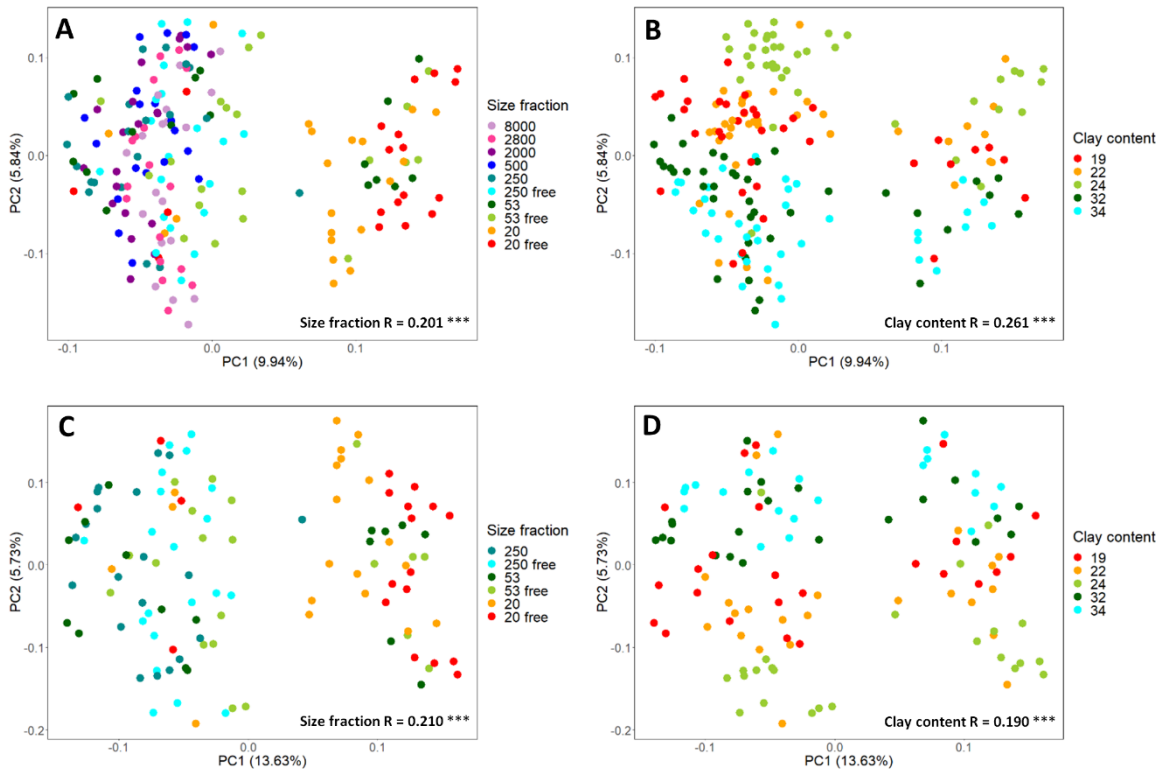

Fig. II-3: Principal component analysis (PCA) showing similarities in bacterial community composition between samples of the five clay contents and ten different aggregate size fractions (A and B) as well as a data subset consisting of the microaggregate fractions only (C and D). Figures include R-values from a statistical evaluation of differences between groups of samples, obtained by analysis of similarity (ANOSIM).

To get a general overview about the variation in bacterial community composition between all samples, we performed a PCA based on CLR transformed data and calculated as complementary approach an NMDS plot based on Bray-Curtis distances. In PCA (Fig.II-3) only a minor part of the variation between samples was explained by the first two axes (9.9 and 5.8%), indicating that no strong influencing factor was associated with the observed variation between all samples. Nevertheless, a certain separation of samples according to size fraction can be seen along the first axis. Especially the smallest microaggregates <20 µm and to some extent those <53 µm are separated from larger aggregates. Along the second axis samples are to some extent separated by clay content, though not systematically with increasing clay content, indicating that there is no consistent variation due to clay content in the overall dataset. A statistical verification of the effect of these two factors by ANOSIM confirmed a small but significant explanatory power of both, clay content (R = 0.26, p = 0.001) and size fraction (R =

0.20, p = 0.001) (Fig.II-3). The influence of the two parameters was also evident in the alternatively calculated NMDS plot (Suppl. Fig. II-1). Likewise as in the PCA plot, the small microaggregates clustered together and were slightly separated from the larger size fractions. The statistical verification based on ANOSIM revealed again slight but significant effects of size fraction (R = 0.20, p = 0.001) and clay content (R = 0.18, p = 0.001), thus confirming the PCA based findings.

To assess the impact of aggregate size fraction and clay content on microbial community composition more specifically, a constraint analysis, i.e. variation partitioning with redundancy analysis (RDA), was performed and showed a weak influence of 10.8% for the size fraction and of 9.3% for the clay content, while the major fraction of the observed variation in bacterial community composition across all samples remained unexplained with residuals of 80.5% (Suppl. Fig. II-3). This is in good agreement with the PCA and NMDS results.

Tab. II-1: Effect of aggregate size fraction on bacterial community composition in soils of different clay content, assessed based on an analysis of similarity (ANOSIM).

Subset: Clay content [%]	ANOSIM R Euclidian	ANOSIM R Bray-Curtis
19	0.186 *	0.193 **
22	0.162 *	0.166 *
24	0.362 ***	0.371 ***
32	0.233 ***	0.280 ***
34	0.441 ***	0.517 ***

To further evaluate the relevance of aggregate size fraction in dependence on soil clay content, the dataset was divided into subsets of samples according to clay content. This allowed us to compare the impact of aggregate size on bacterial community composition in soils of different clay content independently. A significant effect of the aggregate size fraction was observed in the soils of each clay content, with R-values ranging from 0.162 to 0.441 when differences between samples were described by Euclidian distances calculated from CLR-transformed data and from 0.166 to 0.517 for data based on the Bray-Curtis distance matrix (Tab. II-1). There was a rough trend towards higher R-values, i.e. more differences in dependence on aggregate size, in soils of higher clay content, especially for the data analysis based on the Bray-Curtis algorithm. A look at individual PCA plots (Suppl. Fig. II-4) revealed that the

differences were in all cases related to a distinction between smaller and larger microaggregates. At low clay contents (19 and 22%), only some of the microaggregate samples <20 µm and <53 µm clustered apart from the other aggregate samples. At higher clay contents (32 and 34%), nearly all <53 µm aggregates clustered with the larger aggregates, while there was a clear separation of microaggregates <20 µm, especially in the soil samples with 34% clay content. In these soils, there was a further distinction visible between <20 µm free and occluded microaggregates and a certain grouping of the larger aggregate size fractions. This is reflected by the highest ANOSIM R-values observed for these samples (Tab. II-1).

Tab. II-2: Effect of clay content on bacterial community composition in data subsets of different aggregate size fractions, assessed based on an analysis of similarity (ANOSIM).

	ANOSIM R Euclidian	**ANOSIM R Bray-Curtis**
Subset: Macroaggregates		
8 - 2.8 mm	0.484 ***	0.452 ***
2.8 - 2 mm	0.479 ***	0.466 ***
2000 - 500 µm	0.436 ***	0.531 ***
500 - 250 µm	0.189 **	0.308 **
Subset: Microaggregates		
250 - 53 µm	0.367 ***	0.235 **
53 - 20 µm	0.144	0.072
<20 µm	0.136 *	0.157 *
250 - 53 µm free	0.393 ***	0.257 *
53 - 20 µm free	0.389 **	0.159 *
<20 µm free	0.275 **	0.376 ***

We also assessed the effect of the soil clay content within each aggregate size fraction. This revealed a significant influence of clay in most of the individual fractions (Tab. II-2), although the impact strength remained low to moderate with R-values of 0.136 to 0.484 for the dataset based on Euclidean distances. Differences in community composition related to the soil clay content were more evident in the macroaggregate fractions >500 µm than in the aggregate

fractions <500 µm. An inspection of PCA and NMDS plots set up for each aggregate size fraction individually (plots not shown) did not reveal a consistent change in community composition of the individual samples according to an increasing clay content, indicating that clay content does not induce the same systematic changes in all different aggregate size fractions.

3.3 Influence of microaggregate localization in the soil matrix on bacterial community composition

As we had different types of microaggregates, varying not only in size and clay content, but also with respect to their localization in the soil as free units or occluded within water-stable macroaggregates, we analyzed the influence of these three factors comparatively in more detail in a reduced dataset including only all different microaggregate fractions. According to PCA, the smallest microaggregates <20 µm were quite well separated from larger microaggregates along the first axis, which covered 13.6% of the variation (Fig.II-3). Differences due to clay content were primarily reflected by the second axis (5.7%), while a further clear clustering due to aggregate location in the soil matrix was not very evident. Such a further separation of free versus occluded microaggregates was most evident in the smallest size fraction (<20 µm). The general findings were confirmed by ANOSIM, which revealed almost no differences between the free and occluded microaggregates, while the effects of clay content and size fraction were evident, but again not very pronounced according to the R-values between 0.127 and 0.237 (Tab. II-3). Results of a variation partitioning analysis performed for this microaggregate dataset (Suppl. Fig. II-3 B) supports these findings, as it shows that the localization of the microaggregates within or outside of stable macroaggregates explains only 1% of the variation in bacterial community composition between all different microaggregates.

Tab. II-3: Effect of clay content, size fraction and free or occluded status on bacterial community composition of microaggregates, based on an analysis of similarity (ANOSIM).

Subset: Microaggregates	ANOSIM R Euclidian	ANOSIM R Bray-Curtis
Clay content	0.190 ***	0.127 ***
Size fraction	0.210 ***	0.237 ***
Free vs occluded	0.020	0.019

3.4 Identity of the soil bacteria and identification of indicator taxa in dependence on aggregate size

The community compositional analysis revealed that *Proteobacteria* (30.5%) and *Acidobacteria* (16.8%) made up the largest proportion of the bacterial community. Phyla with lower abundances were *Actinobacteria* (9.5%), *Verrucomicrobia* (7.8%), *Bacteroidetes* (4.4%), *Planctomycetes* (4.2%), *Firmicutes* (3.5%) and *Gemmatimonadetes* (2.4%). The comparative evaluation of their relative abundances in samples from different aggregate size fractions (Fig.II-2 A) or of different clay content (Fig.II-2 B) showed only slight differences. This was also observed at order level (Suppl. Fig. II-1). However, adaptation to a specific habitat is not necessarily manifested at a high taxonomic rank. Thus, we aimed at the identification of OTUs that occur preferentially in specific aggregate size fractions by performing an indicator taxa analysis (Dufrêne and Legendre, 1997). This revealed that the highest number and diversity of indicator taxa occurs in the smallest microaggregate fractions <20 µm and <20 µm free (Fig.II-4; list of all identified indicator OTUs in Suppl. Tab. II-2), while the other size fractions hosted less indicator taxa. In the occluded fraction <20 µm different groups of *Acidobacteria* were predominantly enriched. Besides, different OTUs of the phylum *Bacteroidetes*, candidate division WPS-1 and several taxa representing different unknown bacterial phyla were detected. *Verrucomicrobia*, *Latescibacteria*, *Gemmatimonadetes*, *Proteobacteria*, *Nitrospirae* and *Actinobacteria* were represented by one OTU each. In the free microaggregate fraction <20 µm *Proteobacteria* dominated, followed by *Bacteroidetes*, *Acidobacteria* and taxa of a few unknown bacterial phyla. *Armatimonadetes*, '*Candidatus* Saccharibacteria', candidate division WPS-1 and *Actinobacteria* were represented by a single enriched OTU. In the larger fractions of 2800 µm, 2000 µm and 500 µm different members of the phylum *Proteobacteria* and of some unknown phyla were primarily found to be enriched.

4 Discussion

4.1 Low impact of aggregate size on the soil bacterial community composition at field scale

Microbial community composition in soil is controlled by diverse biotic and abiotic factors. This work focused on the relevance of soil structure, characterized by aggregate size fractions. Soil structure is known to be important for the development and maintenance of microbial diversity in soil by creating different and spatially separated habitats for microorganisms. Soil

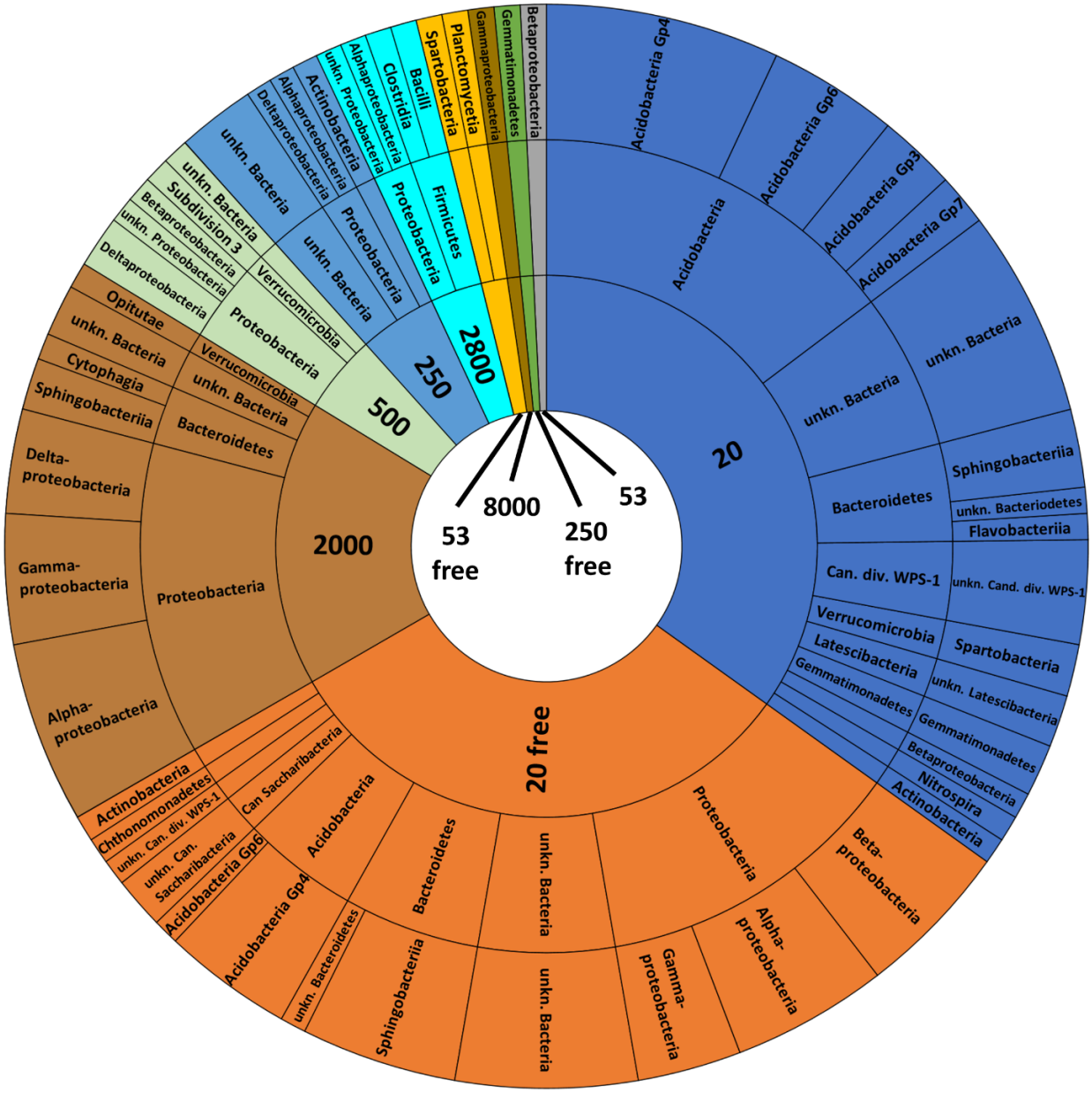

Fig. II-4: Visualization of the indicator taxa identified as significantly enriched within a specific aggregate size fraction. Analysis was performed at OTU level, identified taxa were counted and are presented here at phylum (second circle) and class level (outer circle). The inner circle shows the size fractions in which specific taxa were identified. The size of each segment represents the number of OTUs, the smallest segments represent one OTU. Segments representing aggregate size fractions and the taxonomic groups within each size fraction are arranged according to decreasing frequencies in the number of specific OTUs.

particle aggregation contributes to soil structure formation and thus to the creation of microbial habitats (Chenu and Cosentino, 2011; Wilpiszeski et al., 2019). We extended knowledge about the influence of aggregation on bacterial communities by analyzing aggregates at a higher resolution of size fractions and with a broader range of microaggregate properties. Our results confirm previous studies (e. g. Davinic et al., 2012; Neumann et al., 2013; Sessitsch et al., 2001; Trivedi et al., 2017) showing that aggregate size fraction influences bacterial communities, although this influence was not overly pronounced in the full dataset and primarily seen in beta diversity (Fig.II-3), while alpha diversity remained largely unaffected, except a slight reduction in evenness in the smallest size fraction. Both, PCA and the variation partitioning analysis revealed that approx. 10% of the observed variation in bacterial community composition across all samples was explained by aggregate size. These rather small differences in the bacterial community composition between aggregates of different size demonstrate that aggregate size alone contributes only little to the selection processes that determine microbial community composition at the field scale. Aggregate size does not appear to be very closely linked to aggregate properties that have a strong selective effect on the soil microbial community.

The high amount of unexplained variation (approx. 80%), which was neither related to aggregate size nor to clay content, is likely the result of processes such as drift, dispersal and mutation as well as of various environmental selection processes (Hanson et al., 2012). Environmental factors, which were not covered in this study but may have contributed to variation are soil properties such as pH, nutrient availability as well as mineral and organic matter content and composition (Babin et al., 2013; Carson et al., 2009; Davinic et al., 2012; Delgado-Baquerizo et al., 2018). These factors can directly stimulate or suppress the development of specific microorganisms, or indirectly by modulating physical and chemical properties of soil aggregates and soil structure (Bronick and Lal, 2005; Sey et al., 2008; Six et al., 2004; Vos et al., 2013). Moreover, it has to be kept in mind that aggregates of different size cover traits of soil structure only partly, and that the complete three-dimensional structure that microorganisms face in the intact soil, which is lost upon fractionation, will also be of relevance for soil microbial communities (Young and Crawford, 2004). Microorganisms perceive soil as a coherent three-dimensional matrix consisting of pores and particles in which the respective size of an aggregate appears to play a subordinate role, so that variation in bacterial community composition cannot fully be explained by aggregate size.

4.2 Differences in bacterial community composition are most evident for small microaggregates

We analyzed a number of different aggregate size fractions to evaluate whether specific aggregate sizes or their associated traits contribute particularly to habitat diversification in soil. However, we did not observe that a particular size fraction harbors a higher diversity of bacteria compared to other fractions (Fig.II-1 A), indicating that microbial diversity in soil is not particularly supported by aggregates of a specific size. This observation is consistent with the findings of Väisänen et al. (2005), who likewise found no significant differences in the bacterial community richness when considering different aggregate size fractions. However, other studies noted a formative influence of aggregate size on the bacterial diversity (Davinic et al., 2012; Sessitsch et al., 2001; Trivedi et al., 2017). The cause of these different observations cannot be conclusively assessed, because differences were seen between different size fractions in different studies.

The PCA and NMDS plots as well as the indicator taxa analysis revealed that mainly the smallest aggregate size fraction and to some extent the <53 µm fraction hosted a different microbial community in comparison to the larger size fractions. This is in agreement with some previous studies, where the smallest fraction was also reported to be most distinct (Fox et al., 2018; Neumann et al., 2013; Sessitsch et al., 2001). The smallest size fraction is known to serve as a preferred habitat for microorganisms, based on the finding that bacteria have been preferentially detected in this fraction and that the highest functional diversity has been shown in this fraction (Lagomarsino et al., 2012; Neumann et al., 2013; Ranjard and Richaume, 2001; Zhang et al., 2007). The availability of organic carbon and other nutrients is likely a reason for the distinctiveness of the bacterial community in this fraction. Small microaggregates have been reported to contain mainly recalcitrant carbon compounds, primarily of microbial origin, which are rather difficult to degrade (Plaza et al., 2013). Differences in the quantity and quality of organic carbon were indeed observed in the smaller microaggregates compared to larger microaggregates in the samples from our study site (Krause et al., 2018; Schweitzer et al.; 2019). Likewise, Davinic et al. (2012) observed differences in organic carbon content and quality in the silt and clay fraction compared to other size fractions along with differences in bacterial community composition. It remains currently unclear how exactly organic matter affects microbial life in aggregates of different size. On the one hand, smaller aggregates are considered to better protect organic carbon from degradation (Lehmann et al., 2007; Totsche et al.,

2018), thus microorganisms should be more nutrient limited, likely resulting in limited microbial growth and a preferred enrichment of oligotrophic microorganisms, which are rather slow-growing organisms that are well adapted to nutrient-poor conditions. On the other hand, high microbial activity or extracellular enzyme activity has been reported in association with the smallest fractions (Fansler et al., 2005; Lagomarsino et al., 2012; Nie et al., 2014), which may be seen as indication for good substrate supply.

It should be mentioned in this context that we cannot fully exclude that the wet fractionation procedure contributed to some extent to the observed differences in microbial community composition in the smallest-sized fraction. Cells that dislodge very easily from soil particles in the presence of water may have accumulated in the fraction <20 µm of free microaggregates, while cells that dislodge upon ultrasound may have accumulated in the fraction of occluded microaggregates <20 µm. However, we are confident that the observed differences are not purely the result of the fractionation procedure, because of the very similar clustering of some samples of the <53 µm fraction together with the <20 µm fraction, especially in samples with intermediate clay content (Fig.II-1, Suppl. Fig. II-4). Moreover, studies relying on other fractionation methods have also reported differences between microaggregates and the silt and clay fraction (Davinic et al., 2012; Fox et al., 2018; Sessitsch et al., 2001; Trivedi et al., 2015).

A further clear distinction of the bacterial communities among larger aggregate size fractions was not consistently obvious, i. e. no specific differences were evident along the defined size cut-off between micro- and macroaggregates (i.e. 250 µm) (Suppl. Fig. II-4). This is in agreement with those earlier studies in which strongest differences were also observed for the silt and clay fraction, rather than between micro- and macroaggregates (e.g. Fox et al., 2018; Trivedi et al., 2015). This suggests that bacteria do not necessarily perceive micro- and macroaggregates as distinct units with specific habitats. However, some other studies have reported differences in bacterial community composition between micro- and macroagregates (Bach et al., 2018; Davinic et al., 2012), which suggests that other soil properties may modulate properties between micro- and macroaggregates more strongly and therewith their microbial colonization. Clay content does not contribute to this, as we did not observe more prominent differences between the micro- and macroaggregates in datasets that were subsetted by clay content (Suppl. Fig. II-4).

The different results observed in different studies may to some extent be explained by the fractionation procedures that are applied to obtain the aggregate size fractions. Comparative studies showed that different fractionation procedures can lead to aggregates with slightly different properties and differences in microbial community composition (Blaud et al., 2017; Felde et al., 2020). Community compositional differences can result from the differences in aggregate properties or from specific methodological treatments (as discussed above). We assessed whether consistent findings were primarily reported in those studies that used similar fractionation procedures, but this was not necessarily the case. Studies that were in agreement with ours in reporting major differences at and below the microaggregate scale were all based on wet fractionation methods, but with differences concerning ultrasound treatments and drying steps (Fox et al., 2018, Neumann et al., 2013, Sessitsch et al., 2001). Studies that reported in contrast to our work major differences between macro- and microaggregates were based on different wet as well as dry fractionation procedures (Bach et al., 2018, Bailey et al., 2013, Davinic et al., 2012, Sessitsch et al 2001, Trivedi et al 2017). Similarly, variation in alpha diversity between different aggregate size fractions, which we did not observe, was observed in other studies based on wet fractionation with or without an ultrasound treatment (Davinic et al., 2012, Sessitsch et al., 2001) as well as after dry fractionation (Trivedi et al., 2017). Thus, even though the fractionation method will affect the results to some extent, it is not the major explanation for the discussed differences between different studies.

4.3 Clay content modulates differences in microbial community composition in soil aggregates

The results of this study revealed that clay content per se resulted in little changes in bacterial alpha diversity, while beta diversity was affected at a strength comparable to the impact of aggregate size fraction (Fig.II-1, Fig.II-3). Clay content affected in particular the bacterial colonization of small-sized aggregates, i.e. the fraction <20 µm became more consistently distinct from larger aggregate size fractions with increasing clay content (Fig.II-1, Suppl. Fig. II-4). Several studies that reported clear differences in the smallest-sized aggregate fraction had also high clay contents (> 35%) (Davinic et al., 2012, Sessitsch et al., 2001; Trivedi et al., 2015), which supports our observed trend. However, aggregate studies with soils of low clay content are less frequent, so the weaker distinction in low-clay soils is currently less-well confirmed in the literature.

Clay may affect soil bacteria directly via interactions between cells and surface properties, as e.g. seen in different laboratory studies (Babin et al., 2013; Ding et al., 2013; Huang et al., 2015; Krause et al., 2019). Such interactions can explain the observed differences in the smallest-sized aggregate fraction very well, in which clay particles are particularly accumulated (Krause et al., 2018). This enrichment of clay particles in high-clay soils is possibly linked to an enrichment of specific microbial taxa being preferentially associated to these particles. Besides, clay may affect microbial community composition indirectly via modulated aggregate properties. An increasing clay content leads to increased aggregate stability (Krause et al., 2018; Lado et al., 2004; Wang et al., 2016) and thus also the age of the aggregates tends to increase. The increased stability of aggregates with increasing clay content and the potentially higher age of microaggregates (Totsche et al., 2018) can contribute to the development of more distinct bacterial communities, as these will have better potential for differentiation over longer periods of time, likely along with changes in physical and chemical aggregate properties. This differential development of the bacterial community in relation to clay content was seen in microaggregates, but even more pronounced in macroaggregates (Tab. II-2). These stronger differences in macroaggregates versus microaggregates may be related to the fact that macroaggregates are less stable than microaggregates, so that the stabilizing function of clay is of particular relevance for the differentiation of bacterial communities in the larger aggregates. Schweizer et al. (2019), who analyzed the aggregates from the same study site, showed that a change in clay content had no influence on the soil organic matter composition in different aggregate size fractions. Knowing that the composition of the organic material affects bacterial community composition (Davinic et al., 2012; Delgado-Baquerizo et al., 2018), we conclude that the pronounced differences we observed with increasing clay content were not linked to changes in the composition of organic material, but must be related to other aggregate properties that are modulated by clay content or by the clay content itself.

4.4 Bacterial indicator species identified in different aggregate size fractions

While only little variation was seen in bacterial community composition in dependence on aggregate size fraction at phylum level, indicator taxa analysis identified several OTUs that were specifically enriched, especially in the smallest aggregate size fraction (Fig.II-4). This is in good agreement with the results of the PCA, which showed a differentiation of the smallest aggregate size fraction (Fig.II-3). Among the identified indicator taxa in the <20 µm fraction were a couple of taxa that have been reported to be enriched in the microaggregate and silt

and clay fraction in other studies, including *Acidobacteria* and *Gemmatimonadetes* (Davinic et al 2012, Sessitsch et al 2001) as well as *Actinobacteria* and *Alphaproteobacteria* (Mummey et al., 2006). Characteristic for the free microaggregates <20 µm were besides some *Acidobacteria* various OTUs representing *Proteobacteria* and *Bacteroidetes*. OTUs of the latter two phyla were also seen in some of the macroaggregates, but at lower numbers (Fig.II-4). Many members of these two phyla are known to have a copiotrophic lifestyle, i.e. they are fast-growing organisms that rely on easy accessible carbon sources (Fierer et al., 2007). The enrichment of copiotrophic bacteria in the microaggregate fraction <20 µm is in line with the observation of Krause et al. (2018), who reported higher organic carbon concentrations in the smallest microaggregate fraction compared to the larger microaggregates, especially in the low-clay soils. However, the free and occluded fractions <20 µm did not differ markedly in soil organic carbon contents (Krause et al., 2018). In other studies, the silt and clay fraction has been reported to be notable for the high concentrations of stable nitrogen and organic carbon as well as offering a protective environment for microbial biomass und microbial-derived organic matter (Elliott, 1986). Thus, an enrichment of copiotrophic bacteria appears reasonable and would agree with high microbial and enzymatic activity in microaggregates, which was mentioned earlier.

4.5 Different indicator taxa in free versus occluded microaggregates

Interestingly, different indicator taxa were identified in the occluded microaggregate fraction <20 µm than in the free fraction as well as in comparison to the larger fractions. This seems contradictory to the results of ANOSIM and PCA (Tab. II-3, Fig.II-3 B), which did not reveal significant differences in the occluded versus free microaggregates. However, it can be explained by the fact that we assessed in ANOSIM differences over the complete size range of microaggregates, whereby all free microaggregates are compared with all occluded microaggregates, while the indicator taxa analyses considered each aggregate size fraction and its status as occluded or free separately.

Several of the indicator taxa that were identified in the occluded aggregate size fraction are known to have a rather oligotrophic lifestyle. This has been reported for members of *Acidobacteria* (Fierer et al., 2007; Kielak et al., 2016), *Verrucomicrobia* (Ma et al., 2018; Navarrete et al., 2015), *Gemmatimonadetes* (Hanada and Sekiguchi, 2014), *Nitrospira* (Daims and

Wagner, 2018) and *Actinobacteria* (Zhang et al., 2016). Thus, the occluded small microaggregates may provide more oligotrophic conditions than other aggregates. This is in good agreement with the fact that in particular the occluded microaggregates exhibit a high stability and resistance to microbial decomposition (Clercq et al., 2015), which is probably related to the organic carbon quality in these microaggregates. This is substantiated by the work of Schweizer et al. (2019), who reported a better preservation of the organic matter in occluded microaggregates being <53 µm (without further size separation of <20 µm) compared to free ones in the samples taken at the same study site. The occluded microaggregates may thus provide a rather stable habitat with limited nutrient availability for microorganisms over longer periods of time. Considering that (occluded) microaggregates are formed within macroaggregates, whose formation in turn is often triggered by organic carbon input, it can be speculated that microaggregates are structures that persist after easy-accessible and easy-to-degrade organic carbon has been consumed within macroaggregates, leading to the enrichment of bacteria that are better adapted to nutrient-poor conditions in the occluded microaggregates over time. Once released from a macroaggregate, the microbiota of the then free small microaggregates may change, before these free microaggregates are again trapped in a macroaggregate, where they undergo another cycle of nutrient depletion over time. The presence of fast-growing copiotrophic organisms in free microaggregates and in larger aggregates and of oligotrophs in occluded microaggregates agrees with Odum's (2014) theory on ecosystem colonization and development, which postulates that mature ecosystems are more stable than younger ecosystems. In the latter, higher growth rates can be expected because nutrient availability is higher. In analogy, microaggregates become older than macroaggregates, as deduced from the age of the carbon in aggregates of different sizes (John et al., 2005; Liao et al., 2006) and the longer turnover time of carbon in microaggregates compared to macroaggregates (Monreal et al., 1997). This appears to be primarily evident in occluded microaggregates.

5 Conclusion

Our study revealed that the bacterial community composition varies with aggregate size, although the influence was rather low at field scale and limited to beta diversity. Likewise, the clay content alone did not explain a substantial amount of variation at this scale, indicating that the microbiota is additionally controlled by a number of other mechanisms at this scale. The impact of aggregation on bacterial community composition became more relevant when

focusing on soils of the same clay content, especially in soils with the highest clay content. Specific interactions between certain bacterial strains and clay particles may lead to these differences, while the organic matter composition in the aggregates appears not to be of relevance, as it was not different in aggregates in dependence on clay content. The stability of the aggregate structures over time may support the development of more distinct communities in aggregates of high-clay soils, especially in macroaggregates. The bacterial communities in the smallest microaggregates (<20 µm), which are known to represent a particular habitat for microorganisms, were most distinct from those in the other aggregate size fractions. An enrichment of different copiotrophic bacteria occurred in the free microaggregates <20 µm, while more oligotrophic bacteria were enriched in the occluded microaggregates, indicating that the location of the microaggregate in the soil structure modulates bacterial life. These differences are likely linked to the quality of available organic matter rather than to the amount. While the analysis of different aggregate fractions provided valuable insight into bacterial colonization at the microscale here, more studies on aggregate fractions as well as on samples maintaining the full soil structure are needed to further unravel the role of aggregation and aggregate traits on microbial habitat formation. Methods that enable the analysis of individual aggregates and their microbial colonization hold promising potential in this context. First evidence comes from the analysis of single sand grains, obtained from a sediment sample (Probandt et al., 2018), or from individual macroaggregates (Bailey et al., 2013), revealing a quite heterogenous colonization of individual aggregates or particles.

III

INITIAL MICROAGGREGATE FORMATION: ASSOCIATION OF MICROORGANISMS TO MONTMORILLONITE-GOE-THITE AGGREGATES UNDER WETTING AND DRYING CY-CLES

Modified on the basis of

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* Danh Biesgen and Lars Krause contributed equally to this publication concerning laboratory work, data analysis, interpretation, and writing the manuscript. As this publication was done in joint efforts, results and text excerpts will be part of both PhD theses

1 Introduction

Soil is one of the most complex ecosystems in our world, hosting highly diverse microbial communities, and soil aggregation is considered to contribute to this complexity. Soil aggregates are often categorized into macroaggregates (>250 µm), soil microaggregates (SMA; < 250 µm) and their building units are represented by small SMA (< 20 µm) (Krause et al., 2018; Totsche et al., 2018). SMA are organo-mineral associations with a relatively high stability and persistence and are strongly linked with soil functions (Totsche et al., 2018). SMA have been identified as a favorable habitat for microorganisms and they are often characterized by a high microbial diversity and activity (Poll et al., 2003; Rabbi et al., 2016; Ranjard and Richaume, 2001; Sessitsch et al., 2001; Sey et al., 2008). However, associations between microorganisms and mineral particles have also been reported to be detrimental for bacterial cells (Cai et al., 2013; Ma et al., 2017), suggesting that microbial life is not sustained by microaggregates in general nor during the process of microaggregate formation.

The complex and dynamic initial formation of the aggregate structure is influenced by biotic and abiotic factors. Microorganisms need to be considered as a relevant biotic factor in the process of initial SMA formation. It is well known that microorganisms associate with mineral particles and microbial residues may act as the nucleus/building unit of SMA by occlusion through phyllosilicates and metal oxides (Chenu and Stotzky, 2002; Kleber et al., 2015; Totsche et al., 2018). Mostly, it is suggested that residual cell wall envelopes and other microbial-derived compounds, which persist after cell death, act as a gluing agent for the formation and stability of small SMA (Miltner et al., 2012; Zhang et al., 1999). Moreover, living microorganisms have been suggested to actively influence aggregation (Watts et al., 2005). Their viability and survival are therefore potentially important factors for aggregate development, but this has not been proven yet.

A microbial property contributing to aggregate formation is the secretion of extracellular polymeric substances (EPS), which are mainly composed of polysaccharides, proteins, nucleic acids, and lipids (Flemming and Wingender, 2010). The EPS are secreted by microorganisms, often in increasing amounts under abiotic stress conditions like drought, elevated temperature or salt levels (Roberson and Firestone, 1992; Sandhya and Ali, 2015). As a natural interface layer with enhanced water holding capacity, EPS are believed to enhance the survival of microorganisms upon exposure to abiotic stresses (Roberson and Firestone, 1992; Sandhya

and Ali, 2015). Additionally, EPS participate in the adhesion forces between microorganisms and mineral surfaces allowing the initial microbial colonization on biotic and abiotic surfaces, which can result in the formation of a three-dimensional network in the form of a biofilm (Flemming and Wingender, 2010). Furthermore, EPS bind to soil particles, promoting the formation of stable aggregates (Chenu, 1993; Chenu and Guérif, 1991; Jaisi et al., 2007; Kleber et al., 2015; Oades, 1993; Sandhya and Ali, 2015). According to these findings, the secretion of these biopolymers may also be a factor for the formation of larger and more stable microaggregates, especially in the presence of abiotic stresses. Haruta and Kanno (2015) highlighted that studies on microorganisms exposed to simultaneous and successive stresses will help to understand how bacteria persist in nature. Desiccation stress is known to cause physiological stress to bacterial cells and leads to physical alterations in their EPS, eventually leading to cell death (Or et al., 2007; Schimel, 2018). Moreover, desiccation is known to affect aggregate stability and in some circumstances causes the breakdown of aggregates (Amézketa, 1999), but it can also stimulate aggregate formation (Tang et al., 2011). Actually, wetting and drying processes are considered as a major driver for the reorientation of aggregate building units and development of a three-dimensional soil structure (Chenu and Cosentino, 2011). It remains unclear whether microaggregates support microbial survival under diverse abiotic stress conditions, including desiccation stress. Furthermore, the spatial distribution of living and dead microorganisms under abiotic stress conditions may be an important factor for the dynamic aggregation process and establishment of bacterial cells on small SMA, being modulated by EPS production.

To address questions related to the formation of small SMAs, reductionist approaches are very useful, as they allow one to focus on the factors of interest and to test specific hypotheses. Thus, aggregate formation has been analyzed based on artificial soils under controlled conditions or in aqueous suspension in multiple studies, as e.g. reviewed by Pronk et al. (2017) and Kleber et al. (2015). Such studies provide valuable information concerning the adhesion of bacterial cells or EPS on soil minerals (e.g. Cai et al., 2013; Cao et al., 2011; Di Lin et al., 2016; Hong et al., 2011; Huang et al., 2015). Most of these studies compared the adsorption of EPS or specific bacterial strains on different mineral surfaces under varying conditions, allowing an assessment of the impact of pH, ionic strength, or temperature on the adsorption process. While bipartite associations have been well studied, knowledge is more limited concerning multipartite associations, i.e. the combination of a biological compound and more than one

mineral. Furthermore, the temporal dynamics during the initial phase of small SMA formation from different mineral and biological components remains largely unknown.

Methodological approaches are available that allow the analysis of microbial interactions with SMA or their building units at the bulk scale or based on visualization techniques at the microscale. However, there is a lack of time resolved knowledge, since the development of new aggregates and its dynamics are difficult to observe *in situ*. With the development of liquid cell transmission electron microscopy (Liao and Zheng, 2016), a new technique is available to investigate early SMA formation in the presence of microorganisms at the nanoscale, but this method is limited to low sample throughput and the risk of applying lethal electron doses on bacteria (Jonge and Peckys, 2016). Non-invasive methods represent a valuable alternative, as aggregate structures and microorganisms remain intact. The utilization of an image-based microparticle detector delivers benefits over methods applied in the past. The detector allows a non-invasive real-time size distribution analysis by detecting a large number of particles in suspension without the need of further sample preparation like drying. Furthermore, the visualization of bacterial cells with fluorescence microscopy is a promising method to study the spatial distribution of microorganisms and analyze their role during initial aggregation. The use of a viability staining kit (Stocks, 2004) even enables an assessment of the viability of individual bacterial cells in association with aggregates. A combination of these methodological approaches enables the tracking of the initial small SMA formation process from different soil model minerals and microorganisms.

The aim of this study was to analyze the initial development of small SMA from the soil model minerals montmorillonite and goethite plus different bacterial strains with different surface properties and varying capabilities of EPS production in an artificial incubation experiment. We performed the experiments in the presence or absence of desiccation stress and elucidated whether microorganisms show better survival under stress conditions when occurring attached to SMA. We hypothesized that (I) different bacterial strains influence the formation of small SMA to different extent and (II) the application of repeated wetting and drying cycles will lead to larger SMA. Furthermore, we hypothesized that (III) the majority of microorganisms associate with microaggregates, and that desiccation stress increases the extent of accumulation. Additionally, a (IV) higher EPS production is expected to stimulate the aggregate formation process and to increase the survival of microorganisms upon desiccation stress. The

initial microaggregate formation process was analyzed in detail during the first days of incubation, while microbial survival was analyzed in detail upon extended desiccation stress after the initial microaggregate formation and microbial colonization phase.

2 Material and methods

2.1 Model components

The aggregation experiments were performed with montmorillonite and goethite as model minerals. Na- montmorillonite was purified from fine granular Na-bentonite (Volclay ® MX-80, Lot. 36316, Minerals Technologies Inc., New York City, USA). For purification 5 g of bentonite were suspended in 1 L of MilliQ water and stirred for 30 min. Carbonates were washed out by adjusting the pH to 5 with 1 M HCl. After reaching a stable pH, the bentonite was centrifuged at 15,344 x g for 25 min at 20 °C and the supernatant was discarded. The Na-allocation was performed by resuspension in 1 L of 1 M NaCl and storage for 72 h at 4 °C. After that, the 1 M NaCl was replaced twice using the same centrifuge parameters and volumes. Excess salts were removed by washing the obtained pellet using MilliQ water and subsequent centrifugation at 15,344 x g for 25 min at 20 °C, being repeated until the electrical conductivity reached < 25 µS cm^{-1}. Isolation of particles < 2 µm was performed by sedimentation of 1 L suspension in glass cylinders according to Stokes' law. The isolation was repeated with the collected supernatant according to Stokes' law using a centrifuge with swing bucket rotor and applying 128 x g for 5 min at 20 °C. After isolation, the suspension was shock frozen in liquid nitrogen and freeze dried for further usage. Aliquots of isolated montmorillonite were suspended in 10 mM NaCl (pH 6.5) and measured via dynamic light scattering (DLS) (Zetasizer Nano, Malvern Instruments, Worcestershire, United Kingdom) confirming a mean diameter of 590 nm (+/-124 nm) and a zeta potential of -38.7 mV (+/- 6.54 mV).

Goethite was synthesized by addition of a 10 M NaOH solution to a 0.5 M $FeCl_3$ solution (pure analyzed $FeCl_3$ x $6H_2O$, Merck KGaA, Darmstadt, Germany) under continuous stirring up to a final pH of 12 (Atkinson et al., 1967). The suspension was kept at 55 °C for 120 h, whereby ferrihydrite was converted into goethite. After adjusting the pH to 6 by addition of 0.1 M HCl, goethite was washed with deionized water by centrifugation and decantation. Aliquots of the isolated goethite were suspended in 10 mM NaCl (pH 6.5) and measured via DLS, confirming a mean diameter of 1068 nm (± 232 nm) and a zeta potential of + 16 mV (± 4.8 mV).

We used *Gordonia alkanivorans* strain MoAcy 2 and two strains of *Pseudomonas protegens,* the wild-type strain CHA0, described by Natsch et al. (1994), and an EPS overproducing mutant strain CHA211 (*mucA*::Tn5 mutant, described by Schnider-Keel et al. (2001)). *G. alkanivorans w*as grown in Tryptic Soy Broth (TSB) and *P. protegens* in ATCC medium 3. The *P. protegens* strain CHA211 was grown in the presence of 25 µg mL^{-1} kanamycin. All strains were cultured in 100 mL of medium under constant shaking (120 rpm) at 30 °C. The liquid cultures were inoculated with 0.1% (v:v) of a pre-culture. Bacterial cells in the stationary growth phase were harvested by centrifugation at 10,000 x g for 10 min and 4 °C, followed by resuspension and washing in 10 mM NaCl solution (pH 6.5). The pelleted cells were resuspended in 10 mL of 10-fold diluted medium, which was diluted with a 10 mM NaCl solution. The cells were stored at 4 °C for 12 h before they were used for aggregation experiments. This was done for two reasons: (I) in this way the cells were pre-adapted to the conditions encountered during the aggregation experiments and (II) the preparation of cells, set-up of the complete aggregation experiment and first sampling would not have been possible within one day. Directly before the start of the aggregation experiments, the cells were washed again with 10 mM NaCl solution to eliminate the diluted culture medium. The cell pellets were suspended in 10 mM NaCl solution to a cell fresh weight concentration of 80 mg mL^{-1} and added to the mineral soil particles in the aggregation experiment. Moreover, the size and surface charge of *P. protegens* CHA0 was determined via DLS, confirming an mean diameter of 1050 nm (± 147 nm) and a zeta potential of -2.20 mV (± 6.21 mV).

2.2 Incubation

The incubation of the bacterial strains with model minerals was performed in 2.5 mL of a 10 mM NaCl solution adjusted to pH 6.5 and containing 20 mg mL^{-1} of montmorillonite, 0.49 mg mL^{-1} of goethite, and 3.21 mg mL^{-1} of microorganisms (either *P. protegens* CHA0, *P. protegens* CHA211, or *G. alkanivorans* MoAcy 2). After the minerals were mixed together, the pH was adjusted and microorganisms were added to the suspensions. No further nutrients were added to mimic a natural bacterial soil habitat with very limited availability of nutrients for bacterial cells in the stationary phase. The amount of minerals and microorganisms was defined based on previous bulk analysis of a German Luvisol at the research station in Scheyern by calculating the mass ratio between clay, iron, and total organic carbon. Suspensions were prepared for incubations with and without wet-dry cycles. Triplicates per treatment were incubated in glass petri dishes (STERIPLAN Petri dish, 237553903, DWK Life

Sciences, Wertheim, Germany) sealed with parafilm at 20 °C on a horizontal shaker (KS-15, Edmund Bühler GmbH, Bodelshausen, Germany) operating at 60 rpm. In the wet-dry treatment the samples passed wet-dry cycles consisting of a 10 h wet period, followed by a 14 h drying period under a sterile bench operating with a laminar flow of 0.4 m s^{-1} (BDK-SB 1500, BDK Luft- und Reinraumtechnik, Sonnenbühl-Genkingen, Germany) (Fig. III-1).

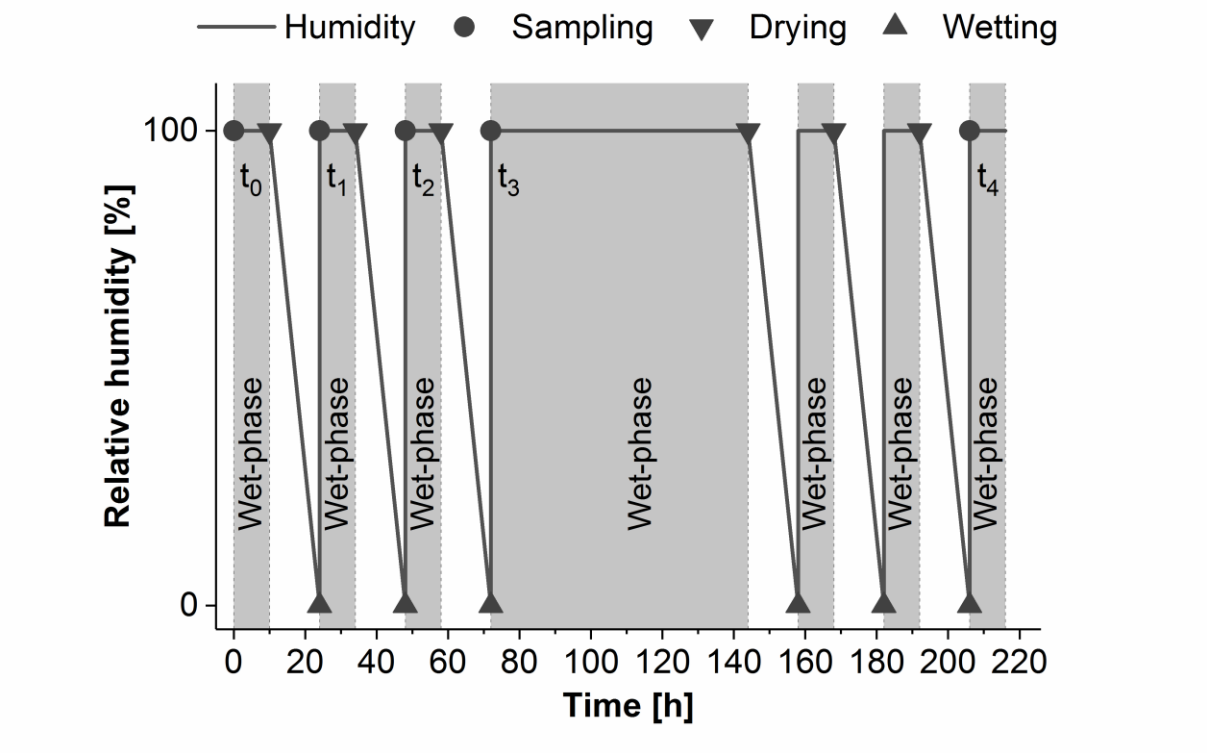

Fig. III-1: Schematic view showing the timeframe of the experimental wetting and drying cycles (specific triangles indicate the beginning of drying and wetting phases, respectively). Overall, 5 time points (t_0 –t_4, dots) were defined for sample analysis at the beginning of the wetting phases.

Drying of the samples was controlled by weight and stopped when no further weight loss was detectable. Samples were re-wetted with MilliQ using the corresponding evaporated volume and were re-suspended by pipetting. In addition, we incubated a control treatment consisting of bacteria in 10 mM NaCl but without montmorillonite and goethite, which was also subjected to wet-dry cycles, to assess the effect of desiccation cycles on bacteria in the absence of minerals. For sample analysis, five time points were defined, in which t_0 represents the beginning of the incubation period immediately after mixing of all components and t_1-t_3 (t_1: 24 h, t_2: 48 h, t_3:72 h) represent rewetted and re-suspended samples after one, two or three drying cycles, respectively (Fig. III-1). Time point t_4 (206 h) was defined after three further wetting and drying cycles (initial wet period for 72 h after t_3, followed by 3 drying-wetting

cycles as described above) in order to observe microbial survival after longer-term exposure to repeated stress treatments. For fluorescence microscopic analyses and determination of colony forming units (CFUs) fresh samples were analyzed, while samples were shock frozen in liquid nitrogen for all further analyses. This widely preserved the natural state of the SMA (Siebers et al., 2018).

2.3 Particle size distribution and spatial arrangement of microaggregates

The size distribution of the small microaggregates in the suspensions were measured with a XPT particle detector (PN3000 XPT Detector, Postnova Analytics GmbH, Landsberg am Lech, Germany). The suspensions were diluted with 10 mM NaCl solution to a final concentration of 1.19 mg mL^{-1}. For each measurement, 250 frames were captured with the XPT software and the number based particle size distribution (PSD) was analyzed according the Waddell disc diameter (diameter of a circle having the same area of the corresponding particle) of detected particles/aggregates. Each experimental replicate was measured based on three technical replicates from which the corresponding mean values were generated.

Shock frozen and freeze dried samples were prepared for scanning electron microscopy (SEM) by spreading them onto a double-sided carbon tape and mounting onto a sample holder. In order to take high magnification micrographs, samples were subjected to a sputter coating, i.e., deposition by sputtering of an approximately 10 nm-thick Au-film onto the sample in order to minimize charging effects for taking high magnification micrographs. The microstructural analysis was obtained using SEM (JEOL 7400f, Oxford Instruments, Abingdon, UK).

2.4 Survival rate and spatial arrangement of microorganisms

To determine the number of culturable cells, two independent 10-fold dilution series were prepared in 10 mM NaCl solution from sample aliquots and 5-µL aliquots of each series were spotted on solidified medium. The agar plates were incubated at 30 °C until visible colonies developed. Colonies were counted in the highest positive dilutions showing a countable number of colonies. Up to 30 colony forming units per spot were counted to calculate the number of living cells mL^{-1} of suspension.

The spatial arrangement of living and dead but intact bacterial cells was evaluated by fluorescence microscopy using a viability staining kit (LIVE/DEAD™ BacLight™, Bacterial Viability Kit

L7012, ThermoFischer Scientific, Waltham, USA). This staining approach allows the differentiation between viable cells with intact membranes and non-viable cells with disrupted membranes. For staining the samples were diluted 1:500 for t_0-t_3 and 1:200 for t_4 in a total volume of 100 µL of 10 mM NaCl solution, mixed with 0.3 µL of the staining mastermix and incubated for 10 min prior to image acquisition. A 10 µL aliquot of the stained sample was transferred onto an agarose coated microscope slide and observed using an Axio Imager M2 epifluorescence microscope with Colibri.2 LED light sources and with a 63x Planapochromat objective (Carl Zeiss, Oberkochen, Germany). The excitation of the fluorophores was conducted at 470 and 550 nm and the emitted florescence was measured at 495 and 570 nm.

The numbers of living and dead bacterial cells, which occurred in association with microaggregates or free-floating in solution, were determined based on 30 microscopic fluorescence images that were taken and underwent an automated image analysis. Bacterial cells were segmented into living and dead cells using the global auto threshold (Yen's threshold) after Yen et al. (1995) in the Fiji package (Schindelin et al., 2012) for ImageJ (v.1.51n, National Institutes of Health, Bethesda, USA). The classic watershed method was used to segment co-located cells into individual cell segments. The images were segmented into aggregates and background through a supervised pixel classification with Ilastik (v.1.30, Ilastik Team) (Sommer et al., 2011). Segmented images were then used to compute the co-localization of living and dead cells either on aggregate surfaces or free-floating. Image overlays were calculated based on the spatial coordinates of aggregates and bacteria to quantify the association of bacterial cells with or without individual aggregates using tools of the R-project (R Core Team 2018, Version 3.4.0).

2.5 Statistical analyses

Statistical analyses were mostly performed based on non-parametric tests, as the criteria for an analysis of variance were not fulfilled, i.e. homogeneity of variance evaluated by Levene's test and normal distribution of the data. Significant differences in the mean aggregate size, derived from particle analysis, were evaluated using either a Friedman-test or a Kruskal-Wallis test, thus comparing whether samples originate from the same distribution at a significance level of $\alpha = 0.05$. This was followed by a post-hoc analysis using Dunn's test with Bonferroni p-value adjustment for the identification of significant differences between different time

points or for particle mixtures with different bacterial strains (OriginPro 2017, Originlab, Massachusetts, USA). To assess differences in the number of culturable cells, a 2-tailed t-test was performed on log-transformed data, comparing the control within the suspension and the wet-dry treatment, respectively, at each time point. For microscopically gained data, differences in bacterial cell numbers in association with aggregates versus cells free in solution were evaluated in the different incubation treatments. Furthermore, differences in aggregate size in relation to the presence or absence of bacteria were assessed. Statistical analysis was performed by a Kruskal-Wallis rank sum test at a significance level of $\alpha = 0.05$ followed by post-hoc pairwise comparisons using Wilcoxon rank sum tests with Bonferroni p-value adjustment (R Core Team 2018, Version 3.4.0). To evaluate differences between aggregated and free cells or living versus dead cells within treatments, Wilcoxon signed rank tests were used. Overall, four levels of significance were defined and are indicated by asterisks (not significant, $P > 0.05$, no symbol; significant, $P \leq 0.05$, *; very significant, $P \leq 0.01$, **; highly significant, $P \leq 0.001$, ***; extremely significant, $P \leq 0.0001$, ****).

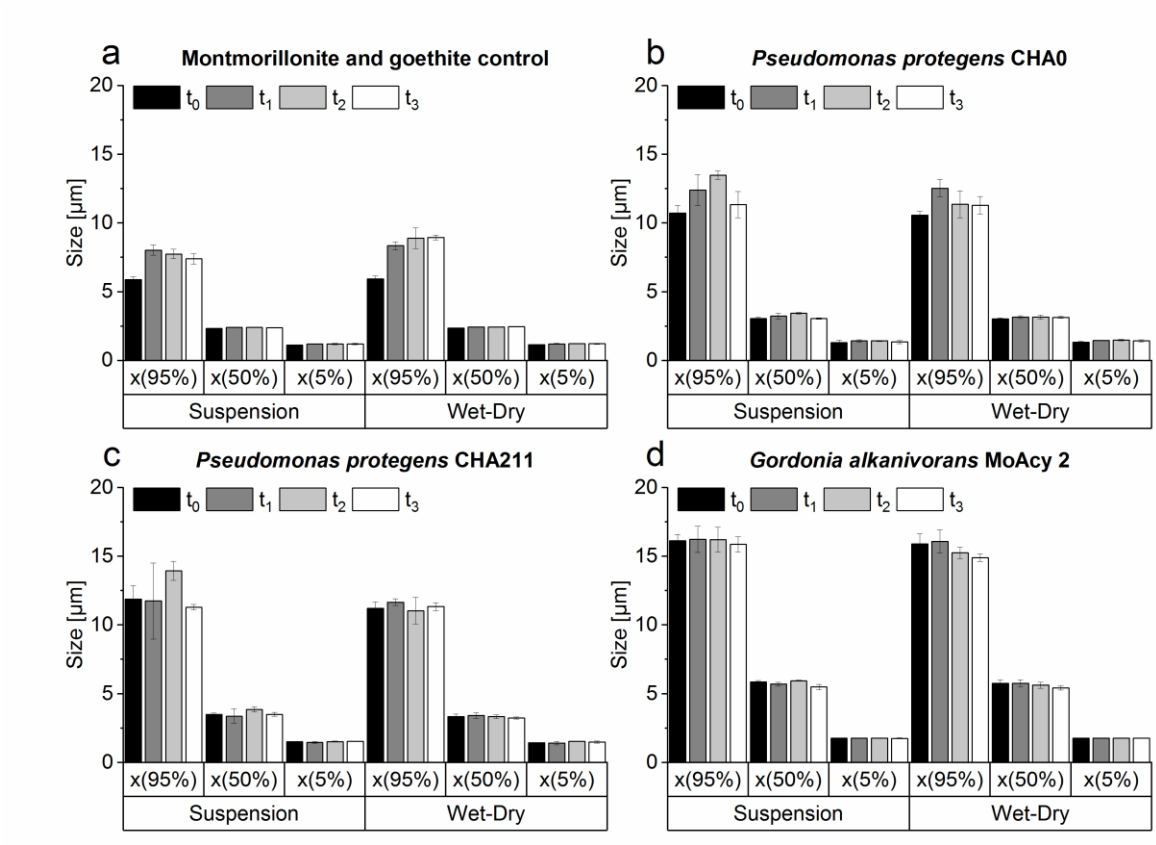

Fig. III-2: Percentiles showing the maximum size of aggregates present in the upper 95% (95th percentile), 50% (median), and lower 5% (5th percentile) of all measured aggregates in montmorillonite/goethite control suspensions (a) and suspensions incubated with microorganisms (b–d).

3 Results

3.1 Aggregate development

During incubation, the particles aggregated to diameters between 1 and 16 µm, thus ranging within the size class of small SMA (Fig. III-2). For all measured distributions there were only minor changes in the median and the 5th percentile, while strongest changes occurred in the 95th percentile (Fig. III-2), i.e., differences between samples were most evident for large aggregates. In comparison to the abiotic control (Fig. III-2a), the addition of microorganisms generally increased the size of the aggregates (Fig. III-2b-d).

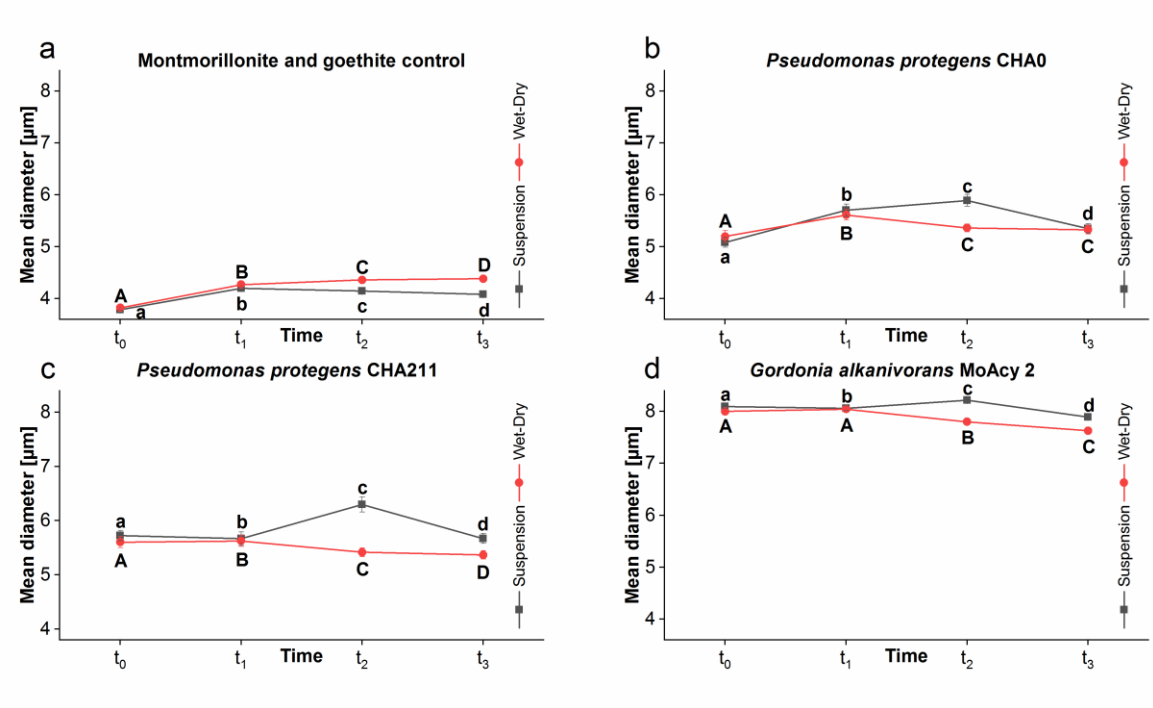

Fig. III-3: Mean diameter and standard error of aggregates observed in different treatments and over time for minerals without microorganisms as control (a), *P. protegens* CHA0 with minerals (b), *P. protegens* CHA211 with minerals (c), and *G. alkanivorans* with minerals (d). Significant differences over time within each treatment are indicated by letters (suspension treatment in lowercase, wet-dry in capital).

Focusing on the temporal dynamic, the negatively charged montmorillonite and positively charged goethite quickly formed aggregates already at t_0, e.g. with a mean diameter of 3.8 µm in the mineral control without microorganisms (Fig. III-3a). The mean size increased significantly from t_0-t_1 in both treatments (permanent suspension and wet-dry treatment). In the suspension treatment, the mean size decreased again after this intensive aggregate formation period, but remained significantly larger compared to the beginning at t_0. Stronger effects were observed in the wet-dry treatment, in which the largest mean diameter of 4.4 µm was

found after three days at t_3. The applied drying and wetting regime supported the development of larger aggregates, as already indicated by the location of the upper percentiles (Fig. III-2).

In the presence of microorganisms, significantly larger aggregate sizes were observed in the suspension treatment at t_2 compared to the aggregates formed in the absence of microorganisms (Fig. III-3b-d). Over all treatments and time points, the formed aggregates were 32% (*P. protegens*) to 93% (*G. alkanivorans*) larger in their mean size than for the abiotic control. The incubation with minerals plus microorganisms led to an initial increase in mean aggregate diameter, followed by a slight decrease. For all aggregates formed in suspension in the presence of microorganisms, the largest mean diameter was reached after two days (t_2). Overall, the mean diameter of aggregates in wet-dry treated samples tended to be smaller than in the corresponding suspension treatments, especially at t_2. In suspension, the diameter increased significantly from t_0 to t_1 and further to t_2, and decreased significantly from t_2 to t_3. In contrast, decreasing diameters were already observed from t_1 to t_2 in the wet-dry treatment. In the suspension treatments of the EPS overproducing mutant *P. protegens* CHA211 larger aggregate sizes were formed than for the wild-type at t_0 and t_3. Likewise as observed for the *Pseudomonas* strains, the incubation with *G. alkanivorans* resulted in the largest aggregates with a mean diameter of 8.2 µm at t_2 when incubated in suspension (Fig. III-3d). While the increase in size from t_0 to t_2 was rather small, the mean diameter became significantly smaller at t_3 than at t_0. The wetting and drying treatment resulted in significantly smaller aggregate diameters at t_2 and t_3 compared to the beginning of the incubation period at t_0.

3.2 Survival of microorganisms

The presence of montmorillonite and goethite decreased the number of culturable *P. protegens* CHA0 cells by 99.99% after the first drying cycle (Fig. III-4a). The number of culturable cells slightly recovered by 0.03% after further wetting cycles from t_1 to t_3. SEM analysis of the wet-dry treated samples revealed that many *P. protegens* cells were disrupted under the given conditions leaving their broken envelopes all over the mineral phase (Suppl. Fig. IX-2). In contrast, the wet-dry treatment under control conditions without minerals did not decrease the number of culturable microorganisms significantly (Fig. III-4a). For the EPS overproducing mutant strain CHA211, montmorillonite and goethite also decreased the number of culturable cells in the wet-dry treatment substantially (Fig. III-4b), but the cultivability remained five

times higher after the first drying cycle compared to the wild-type. In the suspension treatments that did not undergo wetting drying cycles, the number of mutant cells remained constant from t_0-t_3. For *G. alkanivorans* no data could be obtained since the cells had a tendency for homoaggregation, i.e., forming large cell aggregates instead of associating intensively with minerals (see Suppl. Fig. IX-1), which excluded a reliable quantification of colony forming units.

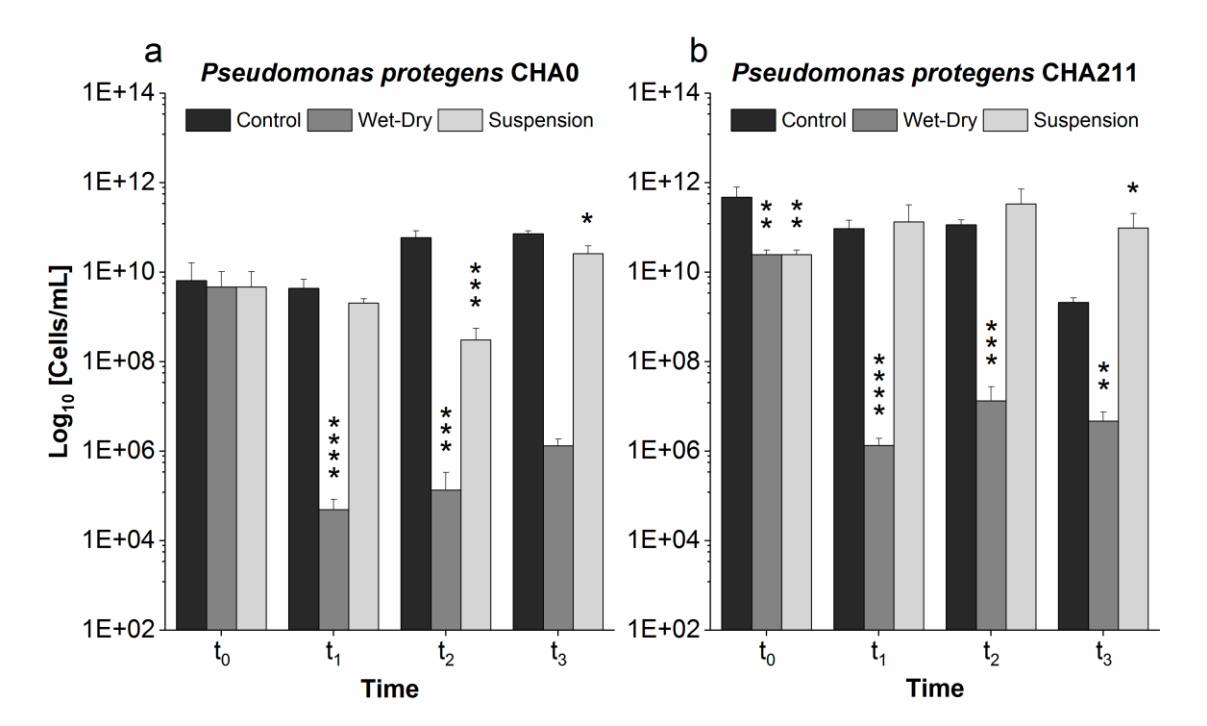

Fig. III-4: Mean number of living cells mL^{-1} of suspension over time for *P. protegens* CHA0 (a) and *P. protegens* CHA211 (b), determined by serial dilution, plating, and counting of colony forming units (CFU). Each strain was exposed to different treatments including control (microorganisms without minerals, subjected to wet-dry cycles), wet-dry (microorganisms with minerals, subjected to wet-dry cycles), and suspension (microorganisms with minerals in permanent suspension). For each time point the significant differences in comparison to the corresponding control conditions are indicated by asterisks.

3.3 Association of *P. protegens* cells with microaggregates

We evaluated the localization of the bacterial cells upon aggregate formation by fluorescence microscopy. This revealed that *P. protegens* cells were rarely associated with aggregates at the beginning of the incubation period, i.e., they occurred mostly free in solution (Fig. III-5a). During the course of incubation, most cells were observed in association with aggregates (Fig. III-5b). The bacterial cell numbers and the number of aggregates with and without microorganisms were determined systematically in all treatments (wet-dry, suspension and control) at t_4, revealing that 2.5% of all aggregates contained microorganisms when applying wet-dry cycles, while 12.5% of the aggregates were associated with bacteria when formed in

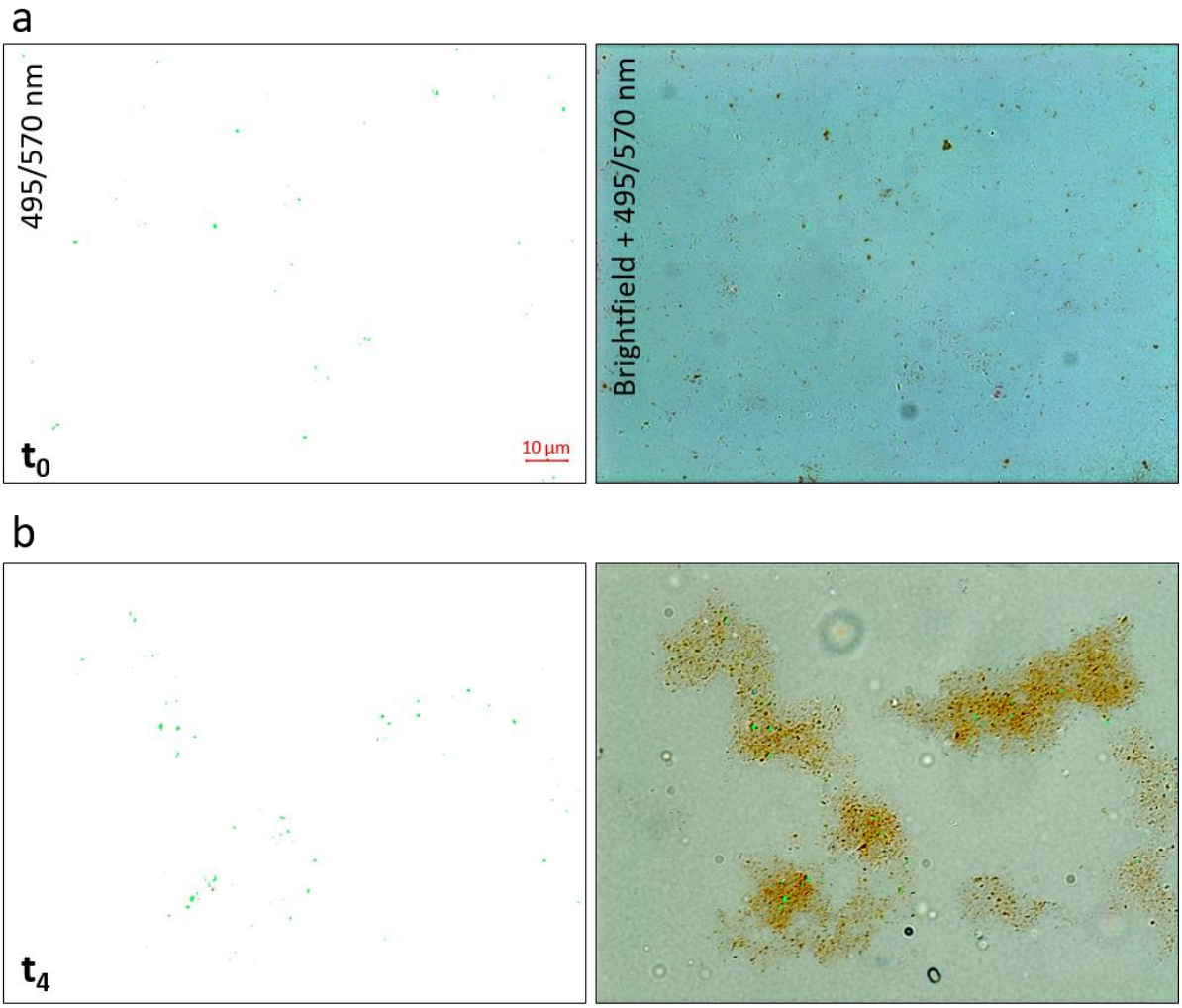

Fig. III-5: Aggregate formation of montmorillonite and goethite in the presence of *P. protegens* CHA0. At the beginning of the incubation period at t_0 the microorganisms were mostly found free in solution (a) while the cells were found almost exclusively in association with aggregates after several wetting and drying cycles at t_4 (b). The images on the left show an overlay of two fluorescent images taken to visualize the viable cells in the green (495 nm) and non-viable cells in the red channel (570 nm). The fluorescent images were combined with a brightfield image showing the microaggregates (right side). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

suspension. For both strains, *P. protegens* CHA0 and CHA211, significantly more cells were associated with microaggregates than occurring free in solution in case of the suspensions as well as for the wet-dry treatment (P always at least < 0.01) (Fig. III-6a-b). The wet-dry treatment decreased the number of aggregate associated cells slightly for both strains in comparison to the cell numbers observed in aggregates in the suspensions ($P < 0.05$), while the number of free microorganisms (CHA0 and CHA211) was substantially reduced in the wet-dry treatment compared to the suspension treatment ($P < 0.0001$) (Fig. III-6a-b). Due to the strong decline in the number of non-aggregate associated cells, the wetting and drying cycles caused the relative amount of aggregate associated *P. protegens* CHA0 cells to reach 98%, while it

was 69% in the suspension (Fig. III-6c-d). Similarly, 95% of the EPS overproducing mutant strain CHA211 occurred predominantly in association with aggregates upon incubation with wetting and drying cycles, while 64% of the cells were found on aggregates in the suspension treatment (Fig. III-6d). Focusing on the living cells, a similar trend was observed as for the total cell numbers (living plus dead), i.e., a 35-fold and 23-fold higher number of CHA0 and CHA211 cells, respectively, was observed in the aggregates than free in solution in the wet-dry treatment ($P < 0.05$), while this difference remained insignificant in the suspension, where cells showed better survival when free in solution. A direct comparison of the cell numbers (total or living only) between the two *P. protegens* strains in the different experimental setups (aggregated or free cells in the wet-dry treatment or suspension) revealed no significant differences. Again, no data were obtained for *G. alkanivorans* because of homoaggregation, which prevented a reliable detection and quantification of individual living and dead cells.

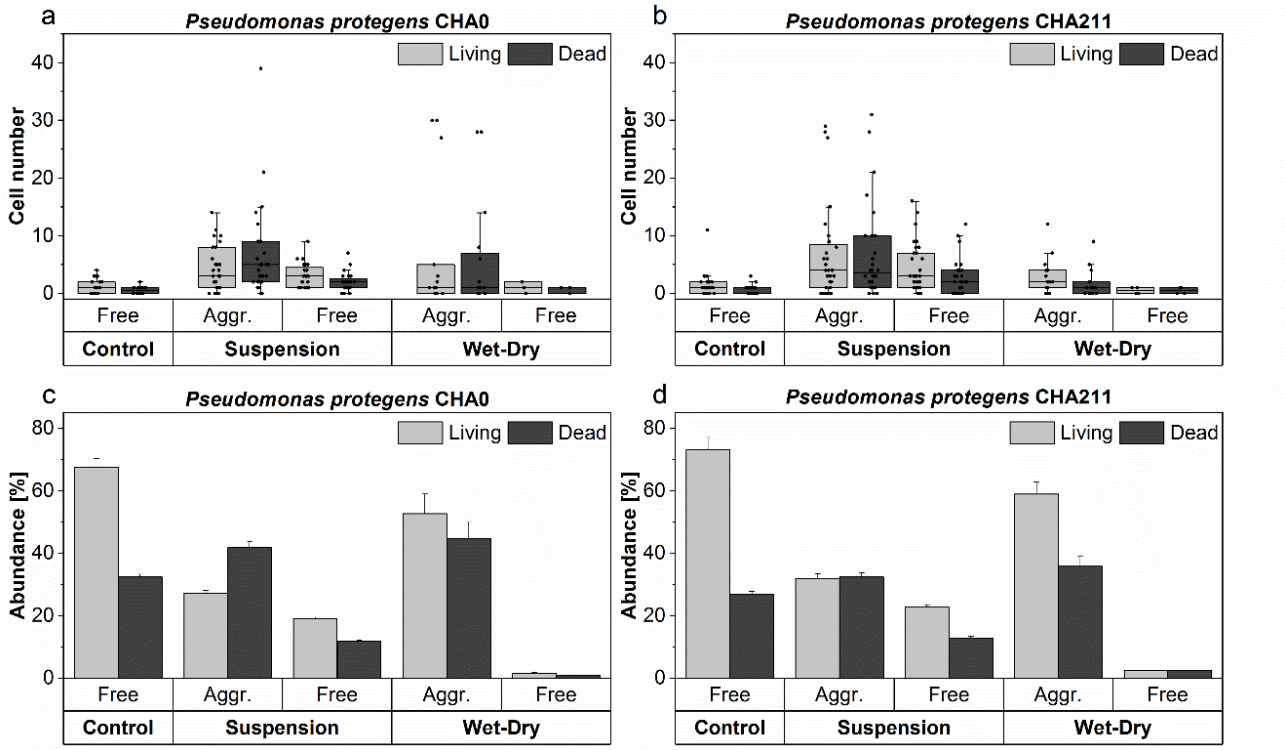

Fig. III-6: Evaluation of fluorescence images obtained by viability stains after t_4. The box-plots show the number of living and dead *P. protegens* CHA0 (a) and CHA211 (b) cells associated with aggregates (Aggr.) or free (Free) in solution within the different treatments. The box plots show the median, the 25th and the 75th percentile (box) and first and 99th percentile (line). Dots represent the results obtained from individual microscopic images to better illustrate the observed variation. Cell numbers were determined in 10 microscopic images per triplicate and per condition. The bar graphs show the relative distribution of detected living and dead cells in each treatment for *P. protegens* CHA0 (c) and CHA211 (d) cells.

The microscopic evaluation of the aggregate size after nine days of incubation at t_4 revealed that the microaggregates harboring bacterial cells were significantly larger than the pure montmorillonite-goethite aggregates ($P < 0.0001$) (Fig. III-7). In the presence of *P. protegens* cells the aggregates were 59- and 72-fold larger after wetting and drying cycles, while aggregate size increased between 12- and 19-fold in the incubations in permanent suspension for the mutant and wild-type strain, respectively. Although the fold-change was slightly higher for the *P. protegens* wild-type strain in comparison to the EPS overproducing mutant strain CHA211, the aggregate size was not significantly different in dependence on the bacterial strain based on the microscopic analysis.

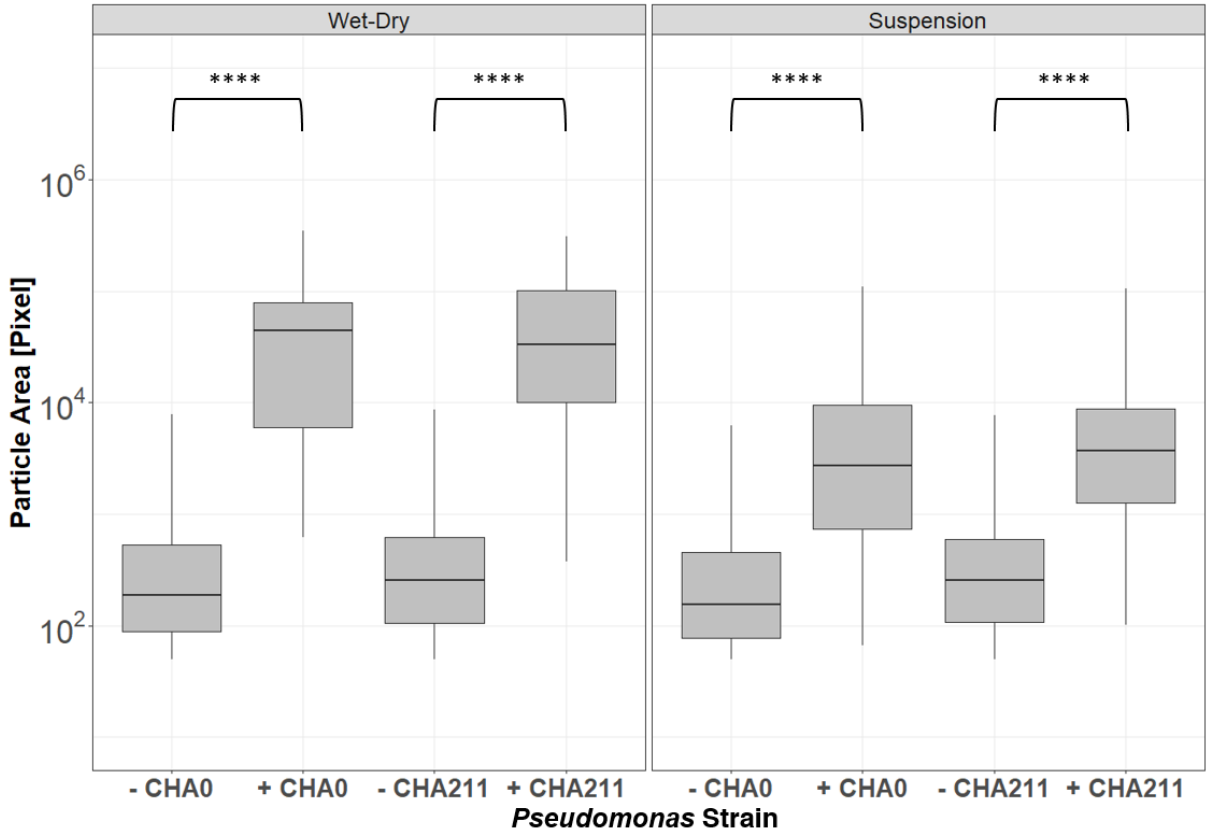

Fig. III-7: Boxplots (with median (line), 25th and 75th quantiles (box), and maximum/minimum values (whiskers)) showing the aggregate size in relation to the presence (+) or absence (–) of aggregate-associated *P. protegens* CHA211 or CHA0 cells at t_4 in the suspension and wet-dry treatments. Pixel size was 0.072 × 0.072 µm. Significant differences are indicated with asterisks.

4 Discussion

The fast development of aggregates within 24 h is a result of strong electrostatic interaction between the negatively charged montmorillonite and positively charged goethite particles. Ferreiro et al. (1995) described a quick aggregation mechanism beginning with mutual particle diffusion, followed by the reduction of the electrical field and bridging of montmorillonite particles between iron hydroxide particles, leading to the association of the two types of minerals with increased particle size. Our observations corroborate the instant and sustained aggregate development after the incubation as described due to electrostatic forces.

Particle size analysis revealed that the presence of microbial cells led to aggregates with 32 to 93% larger mean aggregate sizes due to the association of bacterial cells with aggregated minerals (Fig. III-3). A spatial co-localization of microorganisms with the soil mineral particles was confirmed in particular for larger aggregates based on fluorescence imaging, also indicating that larger aggregates are resulting from the association with microorganisms. However, these colonized aggregates did not form even larger aggregates with each other or with abiotic heteroaggregates over time, probably due to other missing aggregate forming components (gluing agents, cementing agents, building units), their colloidal stability, or the period of time documented in the experiment. The experiment only covered a short time in relation to the timescales of soil development. Furthermore, the microscopic analysis revealed that aggregates containing microorganisms just account for 2.5% of the aggregates from the wet-dry cycles and 12.5% of the aggregates from the suspension. This is in accordance with the findings of the particle analysis, which showed a majority of small aggregates and only a few big aggregates (Fig. III-2). *G. alkanivorans* had a strong tendency of homoaggregation based on its hydrophobic surface properties, provided by long aliphatic chains of mycolic-acids, which are present on the cell surface (Arenskötter et al., 2004; Bendinger et al., 1993; Drzyzga, 2012). These hydrophobic cell surfaces did not allow pronounced interactions between *Gordonia* and montmorillonite or goethite at the single cell level. However, also for this strain we observed an accumulation of soil mineral particles around the bacterial cell homoaggregates (Suppl. Fig. IX-1). Taken together, these findings confirm our hypotheses (I), that microbial strains have an impact on aggregate development and that this is strain-dependent. This strain-dependency is very likely linked to cell surface properties.

The sorption of microorganisms can be very extensive in soils with high clay contents and is mainly based on electrostatic interactions and hydrophobicity (Huang et al., 2005; Sand, 1997). Jiang et al. (2007), for example, showed a fast formation of larger aggregates when colloidal-sized minerals were mixed with *Pseudomonas putida* cells. These bacterial cells had different adsorption affinities with higher affinities to goethite than to montmorillonite, pointing towards a physicochemically determined association preference of bacteria with abiotic SMA building units. Likewise, other studies revealed bacterial species dependent affinities for different mineral surfaces (Ams et al., 2004; Cai et al., 2013; Hong et al., 2011; Huang et al., 2015; Yee et al., 2000; Zheng et al., 2001), suggesting that habitat preferences of bacteria in soil may at least in part be defined by their interactions with mineral particles. This is further supported based on the findings of artificial soil aggregation experiments with varying clay content, where shifts in microbial community composition were observed in dependence on the clay content (Babin et al., 2013; Ding et al., 2013). These differences due to clay content can result from direct interactions of the minerals with the microorganisms or indirectly by changing the microenvironment, e.g. by altered gas or nutrient diffusion processes. The interaction between microbial cells and minerals is not only strain-dependent, also the physiological growth state of microorganisms affects bacterial adhesion (Gargiulo et al., 2007; Grasso et al., 1996) as it influences the surface charge and hydrophobicity of the cells (van Loosdrecht et al., 1987a, 1987b; van Loosdrecht et al., 1990). *Pseudomonas aeruginosa* cells for example have been reported to be less hydrophilic in the stationary phase (Grasso et al., 1996; Grasso and Smets, 1998), and a *Pseudomonas putida* strain showed higher surface adhesion to clay minerals in the stationary phase compared to the exponential growth phase (Wu et al., 2014). We performed the aggregation experiments with cells in the stationary phase, which reflects the prevalent situation in soil, where the majority of soil microbes are not actively growing (Haruta and Kanno, 2015; Lennon and Jones, 2011), due to limited nutrient availability or other unsuitable conditions such as oxygen availability or temperature. Our results show that *P. protegens* and G*. alkanivorans* cells in the stationary phase were forming associations with the mineral aggregates.

Depending on the treatment with or without dry-wet cycles, 69 to 98% of the detected *P. protegens* cells were preferentially associated with the studied soil minerals instead of remaining free-floating in solution, thus confirming hypothesis (III), stating that the majority of microorganisms associate and accumulate with microaggregates and that aggregates appear to

be a preferential habitat for the bacteria in this experimental set-up. When focusing on the treatments that underwent wet-dry cycles, the percentage of cells free in suspension was reduced to 2 and 5% for the *Pseudomonas* wild-type and mutant strain, respectively, reflecting the natural situation in soil quite well. The water limitation clearly forced the attachment of cells to surfaces, likewise as it has been reported for soil microorganisms (Tecon and Or, 2017). The application of desiccation as abiotic stress factor resulted in a reduction of cell numbers within aggregates, but affected in particular the cells that were not aggregate-associated, underlining the sheltering effect of microaggregate structures for bacteria as described before (Chenu and Stotzky, 2002; Deschesne et al., 2007; Lünsdorf et al., 2000). The life-dead staining of the cells confirmed the better survival of the cells within aggregates, i.e., the association of the bacterial cells with aggregates did not result in a dying and conservation of dead cells, but instead supported microbial life under our experimental conditions even after several desiccation events.

Nevertheless, the process of desiccation depleted the number of viable and culturable microorganisms substantially (Fig. III-4 and 6), resulting in the disruption of bacterial cells, which also led to a decrease in mean aggregate size. This disruption was possibly due to mechanical stress, induced by the movement of aggregates and single particles by capillary forces and shrinkage of aggregate components during drying. Furthermore, Ma et al. (2017) and Cai et al. (2013) showed that the interaction between bacteria and goethite can be very strong, resulting in puncturing and injury of single bacterial cells. During drying the injury and lysis of cells may have resulted in a disruption of large aggregates, leading to the 4 to 5% decrease in mean aggregate size over time. Disruption and detachment of bacterial cells due to desiccation is probably leaving microbial residues in the solution (cell envelopes, intracellular components, EPS), as observed in electron microscopic images (Suppl. Fig. IX-2). The liberated intracellular compounds can stabilize the mineral particles again, but this obviously resulted in smaller aggregates compared to the conditions in the suspensions (Fig. III-3). For example, the adsorption of DNA on montmorillonite and goethite (Saeki, K., Kunito, T; Schmidt and Martínez, 2017), and different amino acids on montmorillonite (Dong et al., 2018; Gao et al., 2017) is known to prevent further interaction through steric effects and to diminish the attractive interactions between the mineral surfaces. This mechanism has possibly become more significant after the first drying period, in which the bacterial population size decreased, thus impeding the formation of larger aggregates. In conclusion, the microbial residues have

probably contributed to the aggregation process, in particular under the abiotic desiccation stress. However, our hypothesis (II), stating that repeated wetting and drying cycles in the presence of microorganisms is generally leading to the formation of larger aggregates, has to be specified. We observed an increase in aggregate size due to wetting drying cycles only for those microaggregates that were associated with bacteria (Fig. III-7), indicating that the impact of the stress factor desiccation is of particular relevance if microorganisms are involved in the aggregation process. This increase in size can be attributed to the presence of intact bacterial cells, as well as to envelopes of dead cells (Miltner et al., 2012) or EPS secretion, acting as gluing agents (Chenu, 1993; Sandhya and Ali, 2015).

van Gestel et al. (1996) showed that microorganisms associated to the 2 to 20 µm soil fraction are more susceptible to drying than those associated to coarser size fractions. According to that work, the association of microorganisms with clay minerals does not provide protection against drying, which is in total agreement with our observations after the first drying. However, the total number of microorganisms which survived desiccation is difficult to determine only by cultivation. This is evident from the fact that the microscopic analysis did not reproduce the heavy decline in cell numbers as observed based on the cultivation-based approach. A possible explanation for this discrepancy between methods is the limited cultivability of cells upon exposure to stress. Such a “viable but not culturable” (VBNC) state has been reported for *Pseudomonas* cells upon stress conditions like limited nutrition availability, desiccation or changes in soil pH (Mascher et al., 2000; Mascher et al., 2014; Zhao et al., 2017). Therefore, it is likely that the applied method to quantify culturable bacteria underestimated to some extent the true number of viable microorganisms, as observed by microscopy, which allows the detection of these VBNC cells.

Microbial survival strategies such as the VBNC status (Colwell, 2000) are linked to, e.g., competition, stress, and disturbance (Krause et al., 2014). In a senescent state, microorganisms keep a certain metabolic activity in order to preserve their viability and are protected against stress conditions (Haruta and Kanno, 2015). Such survival strategies can be applied to our observations. From an ecological point of view the VBNC state may contribute to the maintenance of a microbial “seed bank”, since dormancy in the presence of harmful conditions reduces local extinction (Lennon and Jones, 2011). Once better conditions are present, certain regions can be colonized by a rapid recruitment of individual species from the “seed bank” as

a response to the environmental change (Lennon and Jones, 2011). At the small scale, the concept of bacteria taking shelter is described by Lünsdorf et al. (2000), who observed so called “clay hutches” formed around single to few bacterial cells. These “clay hutches” were mainly composed of phyllosilicates, EPS and iron oxides, the same building materials utilized in our experimental setup, but combined under different experimental conditions. Furthermore, it was reported that there were nearly no bacterial cells that were not associated with clay particles, matching our observations that nearly all living bacteria were found in association with aggregates. The authors described the “clay hutches” as minimal soil microhabitat. The results of both studies underline the habitat function of SMA, because survival of bacterial cells under stressed conditions was clearly higher when associated with aggregates. However, further longer-term studies have to be performed to evaluate whether such aggregate associated bacteria, which endure harmful conditions sheltered by SMA, can survive extended periods of time and represent indeed part of the “seed bank” in soil. SMA may exist in soils for a couple of centuries (Totsche et al., 2018), so that microorganisms being entrapped in such an SMA may have to endure very long time spans before being released.

The quantification of the EPS overproducing mutant cells in treatments subjected to desiccation showed a five times higher cell number in comparison to the wild-type, thus supporting our hypothesis (IV), which states that higher EPS production increases the survival of microorganisms upon desiccation stress. This is in line with the literature, where it has been reported that EPS production of *Pseudomonas* species is induced under nutrient deprived and abiotic stress conditions to support survival (Myszka and Czaczyk, 2009; Sandhya and Ali, 2015). Furthermore, a hydration protective effect as well as the upregulation of EPS synthesis genes in response to water limitation is known for *Pseudomonas* species (Chang et al., 2007; van de Mortel and Halverson, 2004). If EPS production was indeed stimulated in our experimental setup, which we did not validate but which may have occurred over the incubation period of several days, e.g. on the expense of lysed cell biomass, it remains unclear why aggregation was not stimulated in the presence of the EPS overproducing mutant strain. Our data showed a comparable effect of the *P. protegens* wild-type and mutant strain on aggregate formation (Fig. III-2 and 7), even though the temporal dynamics were somewhat different (Fig. III-3). Either, the increased EPS production in the mutant strain did not lead to differences in cell surface properties, resulting in comparable interactions with the minerals for both strains. An-

other possible explanation can be derived from the study by Roberson et al. (1993), who describe two types of EPS produced by a *Pseudomonas* strain during desiccation. One type is water-soluble and not strongly associated to bacterial cells whereas the other type is more strongly associated to the cells. The bacteria associated EPS type can account for the better survival of the cells by enclosing the cells and preserving them, whereas the water-soluble EPS type accounts for gluing of soil particles. In case our *Pseudomonas* strains produce different types of EPS as well, the water-soluble EPS type was possibly too much diluted in our experimental set-up to influence initial aggregate formation substantially. It will need further studies to know whether the *Pseudomonas* wild-type and mutant strain used in this study show such differences in EPS production.

5 Conclusion

Based on our observations we conclude that abiotic heteroaggregates between montmorillonite and goethite are formed almost instantly due to electrostatic interaction. The added microorganisms associate and accumulate on the mineral surfaces, promoting the development of larger aggregates. We observed a species-dependent impact of microorganisms on aggregate development, which is likely resulting from different cell surface properties. Furthermore, drying and wetting cycles fostered the association of microorganisms with aggregates and led to the formation of larger microaggregates when microorganisms were colonizing these SMA. However, a reduction in the number of culturable microorganisms during desiccation was followed by a decrease in aggregate size, suggesting that microorganisms or their cell debris contribute to the development of larger aggregates. Moreover, we demonstrated that the SMA support microbial life under adverse environmental conditions such as desiccation, which is a recurring phenomenon in soils that microorganisms have to cope with. Thus, SMA fulfil an important habitat function for microorganisms in soil.

IV

MICROSCOPIC VISUALIZATION OF LIVE/DEAD STAINED BACTERIA ON VARIOUS SOIL MICROAGGREGATES IN NATURAL COLORS

Modified on the basis of the manuscript

Danh Biesgen and Claudia Knief

Manuscript in preparation

1 Introduction

The exploration of the microscopic world and the discovery of bacteria now dates back more than 300 years and is based on the discoveries of Antoni van Leeuwenhoek in 1676 (Porter, 1976). Despite major advances in the visualization of bacteria in the last decades based on the development of a variety of microscopic techniques, the light microscopic in situ visualization of bacteria in soil has always been challenging. However, bacterial life in a complex and heterogeneous environment such as soil can only be fully understood if it is studied at the scale appropriate for individual microorganisms (Cordero and Datta, 2016).

Limitations of microscopy regarding the visualization of microorganisms are mainly due to the heterogeneity as well as opaque nature of the soil and the small size of the bacteria along with their low contrast, which makes their microscopic detection difficult (Li et al., 2004). Fluorescent stains can be applied to improve the visualization of bacteria, but this is often accompanied by the problem of autofluorescence of soil components, resulting in scattered light signals and associated background fluorescence, which aggravates the detection and discrimination of bacterial cells from unspecific signals. Previous approaches for in situ visualization of soil bacteria have often been performed using manually prepared physical thin sections of soil samples (Eickhorst and Tippkötter, 2008a, 2008b; Nunan et al., 2003; Raynaud and Nunan, 2014), which help to overcome these problems partially. However, this requires increased time and effort for the preparation of the samples as well as specific equipment. Besides, context-specific information is reduced to a two-dimensional cross-section.

In soil, the majority of microorganisms is assumed to occur attached to surfaces (Deschesne et al., 2007), and soil aggregates are considered to represent units with an important habitat function for bacteria (Totsche et al., 2018). However, the visualization and localization of individual microbial cells on aggregate surfaces remains a major challenge, although it would provide a distinct view on the distribution and potential habitat preferences of microorganisms in soil.

With the aim to visualize bacterial cells in their natural surrounding and to illustrate (micro) habitat structures on the surface of soil microaggregates, we evaluated microscopic imaging techniques regarding their potential to overcome some of the limitations related to imaging. More specifically, we assessed the potential of structured illumination by using an ApoTome (Carl Zeiss, Oberkochen, Germany) and made use of deconvolution algorithms applied after Z-

stack image acquisition. Both approaches allow the reduction of fluorescent signals from out of focus planes, similar as known from confocal laser scanning microscopy (CLSM). Instead of physical thin sections, as they have often been used in fluorescence microscopy of soil samples, we generated optical thin sections using these techniques.

2 Material and methods

We performed imaging on small microaggregates taken from a microcosm experiment. This was set up with soil from the Ah horizon of the Rotthalmünster research station (Germany), sieved to <250 µm, and incubated for 30 weeks. Before incubation, the soil was spiked with bacterial cultures of the strains *Methylocystis parvus* DWT, *Pseudomonas protegens* CHA0-gfp2, *Gordonia alkanivorans* (DSM 44187) and *Streptomyces viridosporus* (DSM 40243). A detailed description of the experiment can be found in the supplemental material (Chapter X, Appendix D).

Soil samples were stored at 4°C after collection for up to one week until microscopic examination. The particle-associated bacterial cells were stained directly on the microscopic slide using the LIVE/DEAD® BacLight™ Bacterial Viability Kit L7012 (ThermoFisher Scientifc, Waltham, Massachusetts, USA) by following the manufacturer's instructions with modifications regarding the concentration of the dyes. This kit contains a SYTO9 stain for the detection of viable cells and propidium iodide for the detection of dead cells, as the latter dye can only enter cells with defect cell membranes. To mount the samples and prevent rapid bleaching during fluorescence imaging, 50 µl Mowiol/DABCO, a hydrophilic embedding medium based on Mowiol 4-88 (Sigma-Aldrich, St. Louis, USA) and DABCO (1.4-Diazabicyclo-(2.2.2)octane) (Acros Organics, New Jersey, USA) mixed with 0.2 µl of each dye was used. 20 µl of this mounting medium with the two fluorescent dyes was mixed with the soil particles on the slide by carful stirring with the pipette tip to preserve the natural structure of the soil particles, sealed with a cover slip and hardened for 1 h at 42°C in the dark. The samples of the soil to be microscoped were taken randomly without any specific selection. Microscopic image acquisition was performed immediately afterwards using a Zeiss Axio Imager.M2 equipped with an ApoTome.2, a Colibri.2 illumination system, Plan-Apochromat 20x/0.8 and 63x/1.4 objectives, and the filter sets 38 HE eGFP or 43 HE Cy3 for fluorescence imaging. A highly sensitive Axiocam 503 monochrome camera was used for fluorescence image acquisition and an Axiocam 503

color camera for bright field image acquisition (Carl Zeiss, Oberkochen, Germany), which allows the visualization of the natural colors of the soil particles, thus enabling the visual distinction of different soil components. The ZEN 2 pro software (Zeiss) was used for image acquisition and image analysis was done with the additional modules Deconvolution and Extended Focus (Zeiss). Images were taken from individual microaggregates as Z-stacks consisting of 78-151 layers, representing a spatial depth of 21.56 to 40.32 µm. For each image, one Z-stack was captured in the brightfield channel and one Z-stack in each of the fluorescent channels, i.e. eGFP and Cy3. The fluorescence stacks were captured with ApoTome and without ApoTome to evaluate the applicability and suitability of the ApoTome for soil imaging. Subsequently, a deconvolution (Biggs, 2004; Wallace et al., 2001) was performed to eliminate blur and out of focus light from above and below each focal plane using specific deconvolution algorithms (Settings: Algorithm: Contrast, Length scale: 5, Reconstruction: 0.15, Smoothing: 11). The individual images of the Z-stacks were merged into one focused image using an extended depth-of-focus calculation algorithm. Finally, the images of the individual channels (brightfield, eGFP, Cy3) were merged into one composite image. In addition to combining images to form an extended depth-of-focus image, individual images from each focal plane can also be combined to a video sequence, which allows to pass through all focal planes of the microscopic specimen (for the video sequence please refer to the enclosed CD).

3 Results and discussion

Overall, a total of 48 soil particles were investigated. All particles were inspected and analyzed after acquisition. In some cases, it was found that the particles were no longer exploitable due to increased artifact formation and high autofluorescence. However, most of the images showed improved results with the ApoTome (Fig. IV-1 and Suppl. Fig. X-1).

Using the ApoTome for fluorescence image acquisition was of advantage, as illustrated in detail by a direct comparison of images generated with and without ApoTome (Fig. IV-1). The direct comparison of out of the box fluorescence images (Fig. IV-1 A and B) shows that significantly less background fluorescence is observed when using the ApoTome. Most of the signals are from scattered light, derived from out of focus planes, and could be effectively eliminated. These signals result primarily from unspecific binding of dyes to soil components, especially organic matter (e. g. larger green and red signals in the inlet figures of Fig. IV-1 A). While the use of the

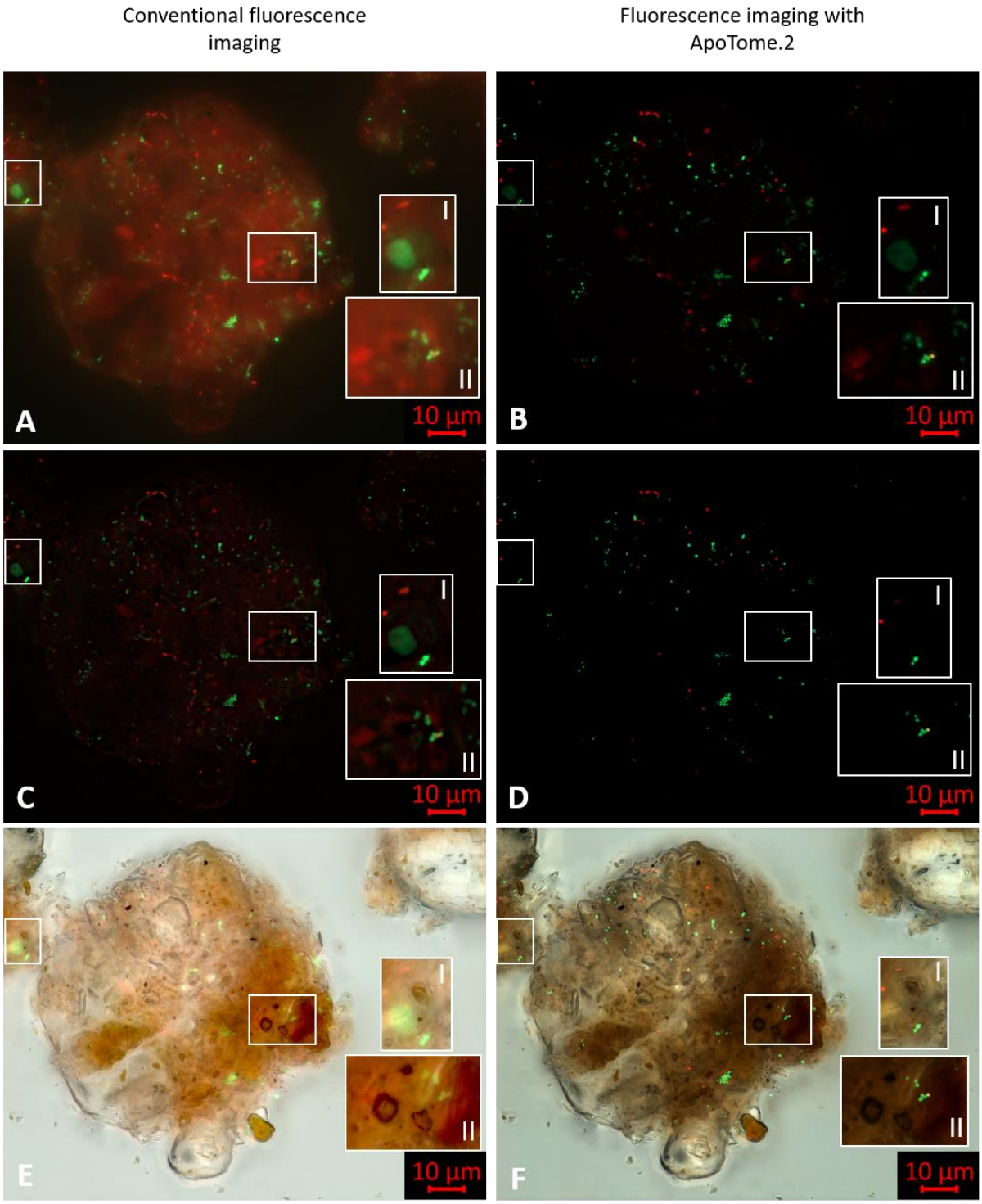

Fig. IV-1: Microscopic images of a soil microaggregate, obtained using different microscopic techniques. The images were generated without (A, C, D) or with (B, D, F) the ApoTome. (A) and (B) show the microscopic images after application of the extended depth-of-focus calculation algorithm. In (C) and (D), deconvolution was applied in addition to reduce blur and out of focus light from above and below the individual focal planes. In (E) and (F), the deconvoluted fluorescence images were merged with brightfield images. Inlaid images I and II show the same close-ups in each of the images. Green fluorescence signals represent vital bacterial cells, whereas red fluorescence signals represent dead bacterial cells.

ApoTome was mostly advantageous, the usefulness became limited when the intensity of the fluorescent signals was very low in all regions of the field of view.

The application of a deconvolution algorithm on the fluorescent image data (Fig. IV-1 C and D) leads to a clear improvement (comparison of Fig. IV-1 A and C). However, artifacts of unspecific staining or due to scattered light remain more intensively visible compared to the unprocessed ApoTome image (inlets in Fig. IV-1 C versus 1 B). When applying the deconvolution algorithm on the ApoTome image, unspecific signals were most efficiently removed (compare inlets between Fig. IV-1 B and D). The deconvoluted ApoTome images were much clearer, which allows a better differentiation of single cells. However, care has to be taken not to lose bacterial signals (see inlet I in Fig. IV-1 C versus D). This demands a careful adjustment of the deconvolution algorithm. When combining the fluorescence images with the generated bright-field images (Fig. IV-1 E and F), unspecific fluorescence signals can superimpose and falsify the final image to some extent, resulting in mixed and false colors (Fig. IV-1 E). This problem was more pronounced for the red fluorescence compared to the green fluorescence images, where non-specific signals were more efficiently eliminated. Besides, color distortion was more perceptibly in brighter colored than darker microaggregate regions (Fig. IV-1 F).
The composite image consisting of the bright field image taken with the color camera in the extended depth-of-field projection and the deconvoluted fluorescence channels recorded with the ApoTome gives an impression of the aggregate in natural colors and also enables the differentiation of bacterial cells against the background of the aggregate reasonably well (Fig. IV-1 F). A requirement for realistic natural color reproduction is that the majority of the unspecific background fluorescence has been successfully eliminated by the ApoTome and deconvolution.

Looking at different individual microaggregates, it becomes evident that each aggregate is unique in its structure and morphology (Fig. IV-2 and Suppl. Fig. X-2 + 3) as well as in its bacterial colonization. In most cases individual microaggregates consisted of one or multiple quartz crystals being more or less densely covered with brownish material, likely representing organic biomass. The distribution and the number of bacteria colonizing the aggregate structures varied considerably. Quite consistently observed was an optical correlation between the occurrence of organic mass and the presence of bacteria, as already described earlier (Ranjard et al., 2000; Ranjard and Richaume, 2001; van Gestel et al., 1996). However, even if there is a

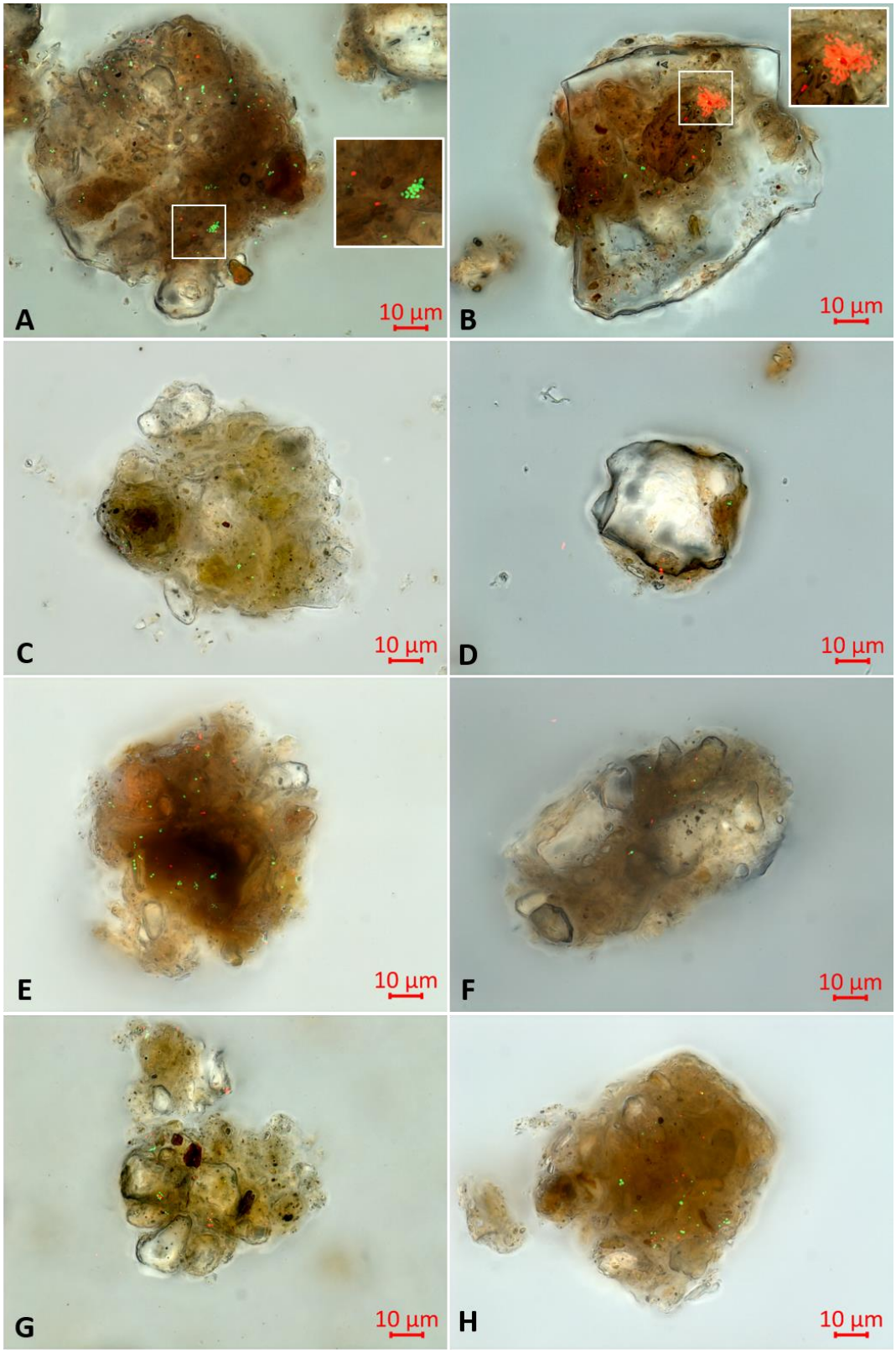

Fig. IV-2: Morphological and compositional heterogeneity as well as variation in microbial colonization of individual microaggregates. Green fluorescent signals represent vital and red fluorescent signals dead bacterial cells. The images were obtained by fluorescence imaging using the ApoTome and subsequent deconvolution. Close-ups of microcolonies of viable (A) or dead (B) bacterial cells are shown in the inlaid images.

correlation between the occurrence of organic material and the presence of bacteria, by far not all of the organic material was colonized, only a very small fraction, which underlines the heterogeneity of the organic matter kinetics (Virto et al., 2010) and reflects the results of Lehmann et al. (2008), according to which organic material is highly heterogeneous in its composition, being of particular relevance at the scale of bacterial habitats. On most microaggregates, we observed single cells, while microcolonies were very rarely observed (Fig. IV-2-A and B). This is in contrast to some literature reports, which suggest the presence of a higher numbers of microcolonies (Grundmann, 2004; Nunan et al., 2003; Vos et al., 2013). Only some microaggregates showed a relatively high number of viable (=green) bacteria or even microcolonies (Fig. IV-2-A, E, H), suggesting that such a microaggregate may represent a hotspot of bacterial activity (Fig. IV-2-A), while other microaggregates were largely free of bacteria (Fig. IV-2-C, D, F and Suppl. Fig. X-2 + 3). Aggregates with predominantly dead (=red) bacterial cells or microcolonies (Fig. IV-2-B) may have encountered a "hot moment" earlier, which seems to be past at the time of imaging. Thus, our images indicate the existence of "hotspots" (Fig. IV-2 A) as well as "hot moments" for bacteria at the microaggregate surface (Kuzyakov and Blagodatskaya, 2015). These phenomena can consequently not only be observed at the large scale, but also at the level of individual microaggregates.

In conclusion, we showed that the application of an ApoTome and of deconvolution algorithms represents a very valuable tool for the improved visualization of bacterial cells on microaggregates by fluorescence microscopy. The techniques can be considered as cost-effective alternative to CLSM and offer the advantage of lower light intensity needed (less bleaching/phototoxicity) and a faster acquisition of single images compared to CLSM. However, care has to be taken not to introduce artifacts by carefully comparing the generated images with the original images seen in the microscope. We show for the first time that it is possible to visualize living and dead bacterial cells in their natural habitat on the surface of microaggregates, which can be visualized in real colors. By capturing a large number of different microaggregates, we are able to visualize the heterogeneity of the soil habitat and show that this variety is accompanied by a diverse colonization by microorganisms.

V
SYNOPSIS

1 Résumé

Soils are the fundamental basis of almost all macroecological life, particularly due to their highly diverse microbial colonization. Although the scientific disciplines of soil science and microbiology have existed for over two centuries, the immense complexity revealed by microscopic observation confronts researchers with the realization that our understanding of the internal structures and their genesis is still largely incomplete. These internal structures, which are common to most terrestrial soils, were characterized by Tisdall and Odes by means of the theoretical aggregate hierarchy model. The aggregated character of soil not only provides it with structure, but also, as a result of its heterogeneously nested and ramified structure, forms the habitat structures and niches that allow the formation of the globally highest bacterial diversity per volumetric unit.

Against this background, the aim of this thesis is to further deepen and illuminate (light microscopically) the understanding of the role of soil bacteria in the colonization of soil aggregates, while also expanding the understanding of how bacteria might already be involved in the initial formation of aggregate structures. This was achieved by performing various experiments with aggregate structures of different size classes and using molecular biological, microscopic, image evaluating and image analytical methods. By applying these diverse methods, it has been possible to gain a better understanding of the role of bacterial communities in interaction with soil aggregates, but also to understand how bacteria themselves in turn influence aggregate structures. Although some questions still remain open, not least due to the immense complexity of soil systems and aggregate structures, and need to be addressed in future studies, the present work provides important answers and food for thought.

For this purpose, the questions already mentioned in the introduction were addressed and answered as follows:

I. **Do varying aggregate properties such as size class, localization in the soil structure as well as variable clay content influence bacterial community composition and shape, allowing the identification and differentiation of specific communities?**

   The heterogeneous physical and chemical composition of soil aggregates provides a variety of potential habitats for microbial life. Based on this, I hypothesized that for

one thing different size classes of microaggregates in addition to their localization (occluded in larger aggregate structures versus free-occurring) have a formative influence on the composition of the bacterial community and therefore there are differences between the distinct aggregate types. For another, increased soil bacterial abundance in quantity and diversity can often be observed in association with clay minerals. Therefore, I hypothesized that a varying clay content in the soil has a formative influence on the composition of the bacterial community at the aggregate level.

My analysis of 10 different aggregate classes in a size range from 8000 to <20 µm showed that aggregate size does have an influence on bacterial community variations, although the influence was rather low at field scale. Similarly, my results regarding the influence of clay content have shown that the influence of clay content has a smaller effect on the variability of the bacterial community than initially assumed. Thereby, each of the two factors, aggregate size and clay content, have an impact of about 10% on the composition of the bacterial community. Concerning differences between free and occluded microaggregates, however, I did not find any significant differences when looking at the total bacterial community. Accordingly, the composition of the bacterial community at the scale of soil aggregates must be influenced by other factors beyond the scope of my analysis that have a strong selective effect. Potential processes which may be involved here are mutation, drift and dispersal as well as other environmental variables.

However, when looking at individual aggregate fractions in detail, I was able to determine that the clay content has a formative influence on the composition of the bacterial community both in the microaggregate fraction, but especially in the macroaggregate fraction. It can be assumed that an increased stabilization of macroaggregates occurs with increasing clay content, which in turn can contribute to the differentiation, formation and maturation of distinct microbial habitats favoring a differentiated bacterial community. Furthermore, it was found that when considering the individual aggregate fractions, clay content affected in particular the bacterial colonization of the smallest fractions. In this case, the fraction <20 µm consistently diverged from the larger fractions with increasing clay content. This smallest fraction can be categorized as belonging to the order of magnitude to which clay minerals are also classified. Although it remains unclear whether clay content by itself exerts this influence, or if clay

content modulates other aggregate properties relevant for the microbial colonization. Further detailed analyses are necessary to clarify this point.

II. **Do certain size categories of soil aggregates provide particular habitats which in turn favor specific bacterial taxa? If this is the case, can particular characteristics of these size categories be identified explaining this?**

Ecological niche differentiation is often not apparent at a high taxonomic rank such as phylum level. In order to identify specific niches being associated with specific aggregate sizes, I performed an indicator taxa analysis to identify OTUs that are characteristic for different aggregate sizes. In doing so, I found that indicator taxa could be discerned in most aggregate fractions, but there was an increased number of them particularly in the smallest aggregate fraction (<20 µm). I could further determine that the identified specific taxa differ insofar that copiotrophic bacteria increased in the free aggregate fraction of the smallest size fraction and oligotrophic organisms became enriched in the occluded aggregate fraction. The difference in the principal lifestyle of the organisms illustrates the preferential colonization of specific microaggregates due to the conditions that shape the habitats of these respective aggregates. In this respect, a possible explanation for the differentiated occurrence of copiotrophic and oligotrophic bacteria might lie in the quality of the available organic carbon in the respective aggregate fraction. This finding indicates that the high bacterial diversity in soils may have its origin in part in association with the smallest soil particles and suggests that the size fraction closest to the size scale of bacteria and their spheres of impact should be given greater consideration in the study of bacterial microniches and habitats in soils.

III. **Do bacteria have a general influence on the initial phase of aggregate formation and do sundry bacterial taxa influence their environment in different ways? Furthermore, do the aggregate structures that form for their part also have an effect on the bacteria that colonize them?**

Until now, there is little knowledge about bacterial involvement in the initial phase of aggregate formation. Whereas it is already known that bacteria have the ability not only to adapt to their environment, but also to actively shape and influence it to a certain degree. To gain more knowledge in this area, I conducted a model experiment

with different bacterial strains and aggregate building units under the impact of wetting and desiccation to include abiotic environmental influences with relevance on aggregate formation. Here, I was able to show that the general participation of bacteria during the initial phase of aggregate formation leads to the formation of larger aggregate structures. Furthermore, I was able to demonstrate that different soil bacteria with various characteristics have a species-dependent effect on aggregate formation, which is partially probably due to different cell surface properties. However, when comparing a bacterial strain capable of EPS overproduction with the wild-type strain, no effect on aggregate size was observed. Nevertheless, it was shown that the increased production of EPS resulted in a better survival compared to bacteria with lower EPS production. In addition to the contribution of bacteria to the aggregate formation process and regarding the effect of aggregates on bacteria, I was able to show that the aggregate structures that were formed had a sheltering effect on the bacteria involved. Bacteria associated with aggregate structures were able to survive periods of desiccation and water deprivation significantly better. These results underline the habitat function that aggregate structures provide for bacteria, but also the share that bacteria actively contribute to the formation of aggregate structures.

IV. **What possibilities do modern light microscopic methods open up in the spatial visualization of complex soil structures and their bacterial colonization?**

Given the heterogeneity and opaque nature of soil structures, light microscopic evaluation of soil microorganisms presents a particular challenge. A majority of soil bacteria are assumed to be located in association with surface structures, however, due to the small size and difficulty of discriminating bacterial cells against the soil background decisively, only few light microscopic analyses of soil bacteria exist to date. This applies likewise for bacteria living associated to soil microaggregates. Since microaggregates represent an important habitat for soil bacteria, my experiments aimed to realize the visualization of soil bacteria and their colonization of aggregate structures. By specific staining and the use of modern techniques of fluorescence microscopy as well as the use of a color camera I succeeded for the first time in visualizing the distribution of living and dead soil bacteria on whole microaggregates, which were imaged in natural colors. In this process, the capturing of image stacks also imparted an impression of the spatial distribution of bacteria on microaggregate surfaces. By comparing a large

number of different microaggregate structures, I was also able to show and represent the high heterogeneity of the soil habitat and to ascertain that the bacterial colonization differs substantially in terms of its distribution from one aggregate to another. Through my experiments, I was able to provide a proof of concept, whereby subsequent studies and analyses should focus on a systematic evaluation of the colonization and its correlation with aggregate properties.

2 Synthesis & Outlook

Terrestrial soils harbor an amount of microbial biomass that can rival the surface biomass of plants and animals (Fierer et al., 2009; Serna-Chavez et al., 2013). As far as microbial biomass is concerned, bacteria and fungi dominate it with 10^2-10^4 times more mass, compared to the other soil microorganisms (protists, archaea, viruses) (Fierer, 2017). Soils itself, in this context, cannot be considered as a single homogeneous environment, but encompasses a large spectrum of diverse and distinct environments, which in turn spawn independent microbial communities. Although these partially isolated environments are spatially only millimeters or even micrometers apart, they can differ significantly in their abiotic and biotic factors, which has an influence on the microbial community as a whole but also on the individual activity as well as abundance of single microorganisms and subcommunities. Here, aggregate structures in general and microaggregates in particular are considered to be one of the key habitats of bacteria (Totsche et al., 2018). Based on this a systematic analysis of aggregate types with a fine resolution in terms of aggregate size classes, clay content and a distinction between occluded and free microaggregates was performed in this thesis. In addition, to gain a better impression of spatiality as well as heterogeneity, microaggregates were observed by microscopy and their bacterial colonization was visualized. Due to the prominent involvement of bacteria in the soil system and its predicted role in aggregate formation, initial aggregate formation and the contribution of bacteria to this process was studied in more detail. The role of bacteria was thus not limited to that of colonizers and inhabitants of the soil system, but their active role in shaping and forming the soil habitat was also investigated in more detail. My studies thus help to shed more light on soil aggregates as habitats for microorganisms and to better understand the role of microorganisms for aggregate formation.

In the following, I will integrate my findings with each other and discuss them in the context of the current state of knowledge.

2.1 Soil aggregates as habitat

Soil aggregates exhibit an ideal habitat structure for microorganisms due to their three-dimensional structure as well as the high degree of heterogeneity and the resulting high potential for the availability of ecological niches. In the following, the findings of this study are compared with existing research findings and the contribution of this research to the advancement of knowledge on the formation of the bacterial community by and through soil aggregate structures is discussed. In addition, the methods used in this study are discussed and suggestions for future analyses are presented.

2.1.1 The effects of abiotic factors

Habitats are affected by a variety of abiotic environmental factors. Although many different abiotic factors and their influences on bacterial soil communities are already known, it is not yet fully known which factors primarily influence bacterial colonization of the soil and to what extent, due to the heterogeneous nature and the large number of different soil types. In this context, it is difficult to determine influential factors at the scale that is appropriate to that of bacteria and their sphere of impact (Hendershot et al., 2017). In this study, therefore, an attempt was made to approach the scale of bacteria by analyzing different aggregate size classes, with the aggregate class of microaggregates in particular approximating the size of bacteria and their sphere of impact. In addition to the influence of the aggregate size class as a first abiotic factor, a systematic investigation of the influence of the clay content as a second abiotic factor was carried out. Clay content was predicted to be a relevant factor, since clay particles are closely associated with bacterial colonization and abundance (Deschesne et al., 2007; Foster, 1988). In this context, this study was the first to systematically analyze the influence of variable clay contents in conjunction with different aggregate size classes and the resulting influence on the bacterial community.

My results showed that both the clay content and the different aggregate size fractions exert less influence on the bacterial community than was initially assumed (Chapter II). Nevertheless, it could be shown that these two factors have an influence on the composition of the microbial community. Since this influence is relatively small, about 10% for each factor, it is

obvious that there must be other factors that influence the composition of the bacterial community either dependent or independent from aggregate sizes. Independent from aggregate size classes, a variety of edaphic factors have been identified that have a formative influence on bacterial community composition and diversity, for example, soil moisture, texture, pH, temperature, the proportion of organic carbon and other nutrients, (Brockett et al., 2012; Cookson et al., 2007; Delgado-Baquerizo et al., 2018; Rousk et al., 2010; Zheng et al., 2019). In particular, pH can be identified as one of the dominant factors in shaping bacterial communities in this regard (Fierer and Jackson, 2006; Rousk et al., 2010; Zhalnina et al., 2015). However, a recent study by Bickel et al. (2019) suggests that pH alone is not a globally valid factor, but that climatic soil water content may be responsible for a significant proportion of soil bacterial diversity and thus may be a covariate of pH. Furthermore, Zheng et al. (2019) studied a variety of different edaphic factors as well as enzyme activities and investigated their combined as well as separate influence. They found that the combined impact of different factors always showed a higher influence than the effect of individual factors. When evaluating the influence of different edaphic factors by the above-mentioned studies and also by a large number of other studies that have dealt with this issue, it should be noted, however, that they have mostly been carried out as bulk soil analyses. Sample analyses at this scale fail to account for the scale and sphere of impact of bacteria, which is why it is at least questionable to what extent these represent the actual influence of these factors or whether these studies do not merely represent the effects on a metacommunity (Cordero and Datta, 2016). This assumption is underlined by the results of this study, showing that clay content affects the bacterial community differently in different aggregate size fractions (Chapter II). Further evidence is derived by Kravchenko et al. (2014), who showed that significant differences in the bacterial community composition were identified depending on whether bulk soil samples or individual aggregate structures of the same soil were analyzed. Furthermore, Hendershot et al. (2017), in a meta-study looking at over 1000 bacterial communities globally and how they are influenced by abiotic environmental factors, found that the abundance and diversity patterns of soil microorganisms do not follow a universal trend. They also conclude that from a bacterial perspective, large scale and coarse resolution surveys of abiotic environmental factors are inadequate to explain fine scale diversity patterns. Although these factors continue to exert a formative influence, it should be noted that this influence is not as pronounced as it has long appeared. Of course, it should be noted that the analysis of some environmental parameters,

such as pH, on a fine scale, such as the level of individual aggregates, remains challenging at present (Buss et al., 2018). Another aspect that should be considered is the assumption that several abiotic factors are influenced differently depending on the scale. For example, when examining the pH value, it is reasonable to assume that it could alter rapidly on a local scale due to the accumulation of protons, which is caused, for example, by microbial metabolic activity. On the other hand, when considering temperature, for example, it cannot be assumed that this changes on a small scale, but changes at larger scales. For instance, the effect of variable carbon, water and oxygen contents could already be observed at the micro scale (König et al., 2020).

Furthermore, in a review on non-free-living, i.e. non-host-associated microorganisms, Langenheder and Lindström (2019) were able to highlight that environmental heterogeneity is of general relevance. However, the authors state that there are many other factors besides these (e.g. dispersal limitation and excess, priority effects in species interactions as well as stochasticity), which are of great and formative relevance depending on the system studied or the prevailing conditions. Lastly, the authors conclude that the individual or combined effects are in turn linked to other properties of the ecosystem, such as the occurrence of scale effects, the overall stability of the system or the overall productivity, whereby all effects vary between the different studies and this even often in a contradictory manner, for which there is as yet no uniform explanation.

In this context, it has been repeatedly shown in the course of this work and in the evaluation and classification of the results that the scale of analysis is of outstanding relevance when evaluating and considering microbial communities in soil systems. Due to this relevance, a detailed discussion will be given in the following section.

2.1.2 The relevance of the spatiotemporal scale

The concept of habitat heterogeneity and the relevance of the spatial scale even in smallest dimensions is not as new as one might initially think. The renowned soil microbiologist Martin Alexander already suggested in 1964 that "microorganisms apparently in the same habitat are, in fact, often exposed to entirely different environmental influences and population pressures. To understand the forces actually affecting the organisms, a microenvironmental concept rather than the gross macroscopic view of interactions must be adopted." However, the technical possibilities at that time were not yet available to carry out analyses at the necessary

scale, which is also critically noted by Alexander (1964). But due to the technical developments of the last decades, which also make the results and approaches of this study possible, one can be confident that the hypothesis, which Alexander (1964) already put forward almost 60 years ago, can now and in the in the near future be further substantiated.

Heterogeneity as result of aggregate composition and the relevance of appropriate scaling

Microorganisms exhibit a high degree of spatial heterogeneity in the distribution and colonization of the soil habitat (e.g. Bahram et al., 2015; Etterma and Wardle, 2002; Franklin and Mills, 2007; Green and Bohannan, 2006). This heterogeneity in distribution is due to the heterogeneous composition and spatial structure of the soil system, whereby in turn the latter can have beneficial effects on the survival of different microorganisms. In the analysis of the biogeography of microorganisms, many different size scales have been considered and differences in the microbial community have always been observed. These range from global and continental scales (e.g. Bahram et al., 2018; Barberán et al., 2012; Fierer and Jackson, 2006) to the landscape scale (e.g. Bru et al., 2011; Pasternak et al., 2013) and even centimeter level (O'Brien et al., 2016) and ultimately to the micrometer level (Szoboszlay and Tebbe, 2021). When comparing soil samples taken only a few centimeters apart, O'Brien et al. (2016) were thus able to show that a remarkable disparity in bacterial community structure already occurs at this scale. This scale-dependent heterogeneity has its origin in the range of mechanisms that influence the formation of microbial communities (Jackson and Fahrig, 2012; Miguet et al., 2016). Therefore, it is highly relevant to consider this circumstance already during the design of the experimental setup and to consider a corresponding scale-sensitive sampling and sample size to be analyzed.

This study was able to meet these demands, whereby the spatial scales were resolved down to the micrometer level. In a first study, the bacterial colonization of different aggregate size classes, which differ in the micrometer range, was analyzed (Chapter II). In a complementary approach, microscopic observation of single microaggregate structures was realized (Chapter IV), which allowed to visualize the surficial bacterial colonization of individual aggregate particles. In addition, the association of minute soil-forming particles and bacterial cells was observed and conclusions were drawn about the behavior of bacterial cells in interaction with the smallest soil building units under controlled conditions (Chapter III). These approaches could thus indicate that bacterial colonization differs even in the micrometer range. On the one hand, this finding has been demonstrated by comparing different aggregate size classes

in pools of aggregates of the same size. On the other hand, this finding was substantiated by microscopic observation of individual aggregate structures. Here it was shown that individual aggregate structures already differ substantially from one another in terms of microscopic appearance and therewith features like morphology and composition. This heterogeneity, observable solely at the optical level, reflects the heterogeneous nature of soil systems and shows that heterogeneity can be detected simply by looking at these soil structures. Based on the morphological and compositional heterogeneity, heterogeneous colonization of aggregate particles can also be inferred, which could also be visualized in this study (Chapter IV). In this respect, the observations of this study at the scale of individual aggregates are in agreement with analyses of Lehndorff et al. (2021), who were able to visualize the elemental composition of microaggregate structures with a resolution accuracy of one square micrometer using electron probe micro analysis (EPMA). In this context, the results of Lehndorff et al. (2021) impressively demonstrate the heterogeneity of aggregate structures with respect to the distribution of C, N, P, Al, Fe, Ca, K, Cl, and Si. In a systematic comparison of 60 aggregate structures, no uniform patterns could be recognized, which would indicate a reproducible composition of aggregate structures. The only correlation found by the authors was the co-occurrence of bacterial C and plant C, as derived from specific element stoichiometries. This correlation also supports the observation made in this study regarding the increased presence of bacterial cells in association with organic matter (Chapter IV). Taken together, these findings indicate that the trend of spatial heterogeneity in bacterial communities ranges from the global scale down to the micrometer scale, where high spatial heterogeneity is seen in the distribution of microbial cells on aggregate surfaces. Furthermore, these results indicate that the composition of aggregate structures appears to have a formative influence on the distribution and abundance of bacterial communities, although the mode of composition of the aggregate structures itself remains unknown.

Spatial heterogeneity as consequence of aggregate structure

Against the background of the high heterogeneity in composition of aggregate structures, if not even to speak of singularity, attention shall be drawn to another special feature of soil aggregates. Soil structures in general and aggregate structures in particular are characterized by an immense surface - compared to the dimension of the volume of an aggregate - due to their multilayered and nested structure as well as the almost infinite possibilities of combina-

tions of their individual components (e.g. Lehndorff et al. 2021). This in turn results in a multiplicity of different potential habitats for microorganisms. This particular characteristic of soil structures makes it almost essential to perform observations and analyses at a microaggregate scale resolution, if not to make it the benchmark when it comes to the understanding of the soil system and the life of microorganisms in soil.

Because of its aggregated structure, the spatial scale is of particular relevance. Due to the aggregation of microparticles, a network of pores and surfaces is formed, resulting in an SSA ranging from 20 to 800 m^2g^{-1} (Bin et al., 2007; Pennell, 2016; Schweizer et al., 2019), depending on the soil type and the amounts of aggregate building blocks involved. Therefore, when analyzing microbial colonization of soil aggregates, it is of particular relevance to be aware of the structural arrangement of aggregates, which again affects the spatiotemporal scale and the importance the latter has for the colonization as well as the impact it has on the development and assembly of microbial communities.

One aspect that the high SSA of aggregate structures contributes to is the multitude of different microhabitats, which enable specialized microbial communities with various characteristics. Therefore, the highest possible degree of consideration and attention to spatial heterogeneity with simultaneous structural conservation is essential to allow the identification of possible synergistic and competitive interactions between different taxa. For example, it has been shown that bacterial co-cultures are capable of achieving significantly higher degradation efficiency compared to each acting separately in monoculture (Cortes-Tolalpa et al., 2017). Moreover, it is proven that the interaction of neighboring cells can influence the metabolic processes of the whole bacterial communities and thus have an impact on nutrient cycling (Ho et al., 2016; Huang et al., 2005). Also, it should be noted that the majority of soil microbiological studies are based on homogenized sample material, which ignores the natural heterogeneity and complexity of soil systems as well as its structural integrity. For example, Kravchenko et al. (2014) showed that there are significant differences in the bacterial community composition when bulk soil samples are compared with individual macroaggregate structures. Thus, the analysis of different aggregate fractions, as realized in this thesis (Chapter II), already represents a significant improvement over the analysis of homogenized sample material in terms of structural conservation.

However, in addition to the occurrence of synergistic effects of directly neighboring organisms, it is also known that the different steps of a larger process occur spatially and structurally

separated from each other (Ranjard and Richaume, 2001). For example, the different steps of the nitrogen cycle occur in different size fractions of the soil, with active nitrifiers predominantly found in the 2-20 µm fraction (Lensi et al., 1995) and nitrogen-fixing bacteria most commonly detected in the < 2 µm clay fraction (Chotte et al., 2002). Differences in the bacterial community, which have their origin in structural heterogeneities of aggregates, could also be found in the context of this study. By performing an indicator taxa analysis for a detailed comparison of the different aggregate size fractions, I found that the majority of indicator taxa were present in the smallest aggregate fraction (Chapter II). In addition, the localization of the microaggregate fractions within or outside of stable macroaggregates and hence also structural variations, made a difference. Thus, it was shown that predominantly oligotrophic indicator taxa could be identified in the occluded aggregates, whereas mostly copiotrophic indicator taxa could be identified in the free fraction. Thus, these results indicate that, on the one hand, the structural composition and spatial scale influence the general composition of the bacterial community, but on the other hand, the biology as well as the lifestyle of the bacteria are also affected.

A further aspect that is of relevance in the context of the high SSA of aggregate structures and should be taken into account is the topography of soil aggregates. The latter is also caused by the heterogeneous structural arrangement and could also have an influence on the bacterial colonization of aggregate surfaces. A first step in capturing and assessing the topography of aggregates was made with the help of the microscopic study (Chapter IV) of this thesis. In this study, the aggregate surface including the bacterial colonization was captured by optical thin sections. Arranging these thin sections into a video sequence reveals the approximate topography of the aggregate along with the bacterial colonization. A next step would be to analyze and determine the topography of soil aggregates and their influence on bacterial colonization more accurately and to investigate whether there are topographically preferred habitats for the microbial colonization of aggregate surfaces.

Microbial adaptation to spatial conditions

Another aspect to be considered in spatial analysis at microscopic resolution is the morphological adaptability of bacterial cells. It can be assumed that bacterial cells in natural systems and under the influence of a variety of selection factors also behave differently in morphological terms compared to cells cultivated in the laboratory under optimum conditions. Electron microscopic analyses of soil samples show that the size and structure of bacterial cells in soil

systems are often in the size range < 1µm, and a majority of the observed cells were actually so-called "dwarf" cells with a diameter of < 0.5 µm (Bae et al., 1972; Bae and Casida, 1973; Balkwill and Casida, 1973). In addition, it was shown that bacterial cells are able to adapt their morphology to the surrounding environment. They were observed to be able to squeeze into constrictions that were less than half of their own diameter, enabling them to adapt their morphology to given shapes and to access structures at the nanometer scale (Hol and Dekker, 2014). This finding is also important when evaluating fluorescence microscopy images of bacteria associated with soil particles, as based on this finding, exclusion of cells due to small cell size or irregular morphology must be questioned.

The importance of the temporal dimension

In addition to the spatial aspect, the temporal aspect is also of great relevance in the analysis of microbial communities in soil systems. Seasonal and climatic influences have the potential to profoundly shape or alter the bacterial community (e.g. Lipson and Schmidt, 2004; Rodriguez et al., 2019; Smit et al., 2001; Stevenson et al., 2014). Just as climatic influence has been shown to be formative at global and continental scales, it can be assumed that this is also the case at microscopic scales. Here, the seasonal climate in the form of temperature differences as well as the daily weather in the form of precipitation or extreme drought can be formative. This can manifest itself in the form of the prevailing temperature but also in the availability or lack of water and oxygen. The effects of the daily weather or the seasonal climate do not only affect the entire soil body, but also influence aggregate structures down to the micrometer scale, e.g. when pores are filled with water and thus can lead to a lack of oxygen or dry out and thus cause a lack of water. Under this aspect, it is therefore also very important to take into account the temporal component in the process of sampling, since different conditions may prevail in the soil depending on the point of time, which in turn may have a determining effect on the bacterial community.

An indication of the short-term impact of abiotic circumstances and events that shape the bacterial community is also provided by the model experiment on initial aggregate formation (Chapter III). In this experiment, wet-dry cycles were used to simulate the temporal fluctuation of abiotic factors in soils, as they often occur during the course of a year or even a day. Here, it was observed that at the beginning of the experiment, after the first wet-dry cycle, there was a decrease in bacterial cell number, which then recovered slightly over time during the

experiment. It was concluded that a large number of bacteria died off due to the abiotic influences, a fact that was optically proven, since a large number of cell envelopes could be made out when observed under an electron microscope. Since the experiment was nutrient-limited, it can be assumed that the renewed slight increase in bacterial cell numbers was supported by nutrients released from lysed cells. Such processes are also conceivable in natural systems, when there is a decimation of certain parts of the bacterial community due to abiotic influences or other disturbances. The nutrients thus released can then be used by other parts of the community (which may have been less active or even inactive up to this point), which in turn can lead to their proliferation or activation. In this process, previously present bacteria disappear as they are dissolved and replaced by others, and in turn other species come to the fore. This aspect of the temporal component must be considered when analyzing and evaluating bacterial communities in natural systems. An example of the observation of such dynamics in natural soil samples was provided by Nunes et al. (2017), who observed that the naturally predominant bacterial community was replaced by a bacterial community of a different composition after the strong impact of an abiotic factor.

Another indication of the relevance of the temporal component at the microscopic scale can also be shown by the microscopic results of this study. In the microscopic analysis of individual microaggregates, it was striking how variably the individual aggregates were populated. One aggregate that housed a comparatively large colony of dead bacterial cells was particularly noticeable (Chapter IV). This observation suggests that this specific region of the aggregate surface represented a habitat for living cells earlier. However, before the time of observation, an unknown factor caused the cells to die. Microbial activity in soils is dependent on a variety of environmental factors (Chapter V, 2.1.1), which in turn causes most soil bacteria to be either in a minimally active or even dormant state (Blagodatskaya and Kuzyakov, 2013) until environmental conditions change in their favor and they become active again, enabling potentially even growth. Kuzyakov and Blagodatskaya (2015) describe these mostly brief periods of activity as microbial “hot moments”, which in most cases have only a short and infrequent time frame, but can also result in bacterial activity being initiated over longer periods of time. The observations of this study show that the concept of hot moments, although not often observed (which may be due to the fact that these events often have only a short time window as mentioned before), can also be found on the microscopic level. Moreover, these results show that heterogeneity is not only a spatiotemporal aspect, but that there is also a close

spatial and temporal relationship between life-promoting and life-hostile conditions. Therefore, the occurrence of such hot moments and the overall temporal dimension are of non-negligible relevance and should be taken into account, as well as the spatial scale already mentioned, starting from the planning of studies, through sampling, to storage and processing of samples.

Prospects for the future investigation of spatial heterogeneity

Future research is needed to better understand the colonization of aggregate structures by soil bacteria by analyzing the formation dynamics of bacterial communities as well as the influence of different abiotic factors including nutrient availability. This could be realized by artificial aggregate structures created in 3D printing. In this context, aggregate structures could be created from different components in 3D printing, and thus different nutrient distributions could also be achieved. A particular attraction of using 3D printing techniques is replicability. Thus, a large number of replicates could be created, which in turn could be exposed to different abiotic and biotic factors, making it easier to understand the effect of these individual factors. In addition, identical structures in replicates could be provided to the same bacteria to analyze how bacterial dynamics evolve under the same basic conditions and whether there are already variations here. Furthermore, the use of 3D printing techniques could be further extended and complemented by nanotechnology, which enables the creations of nanoscale structures (Hol and Dekker, 2014).

2.1.3 Significance of the aggregate hierarchy concept from a microbiological perspective

The investigations of this study are based on the basic idea of the aggregate hierarchy model according to Tisdall and Oades (1982). After working with sample material obtained and analyzed on the basis of this model, however, some critical questions arise regarding the application of primarily pedological methods in answering microbiological questions.

To begin with, the aggregate hierarchy model must be scrutinized critically from a microbiological point of view regarding its basic methodology. The extraction of the different aggregate size classes is based on the application of ultrasound, which Tisdall and Oades in turn adopted from Edwards and Bremner (1964, 1967). The research interest of Edwards and Bremner was focused on the stability of soil structures and the method of choice was the use of ultrasound to investigate how soil performs under the influence of ultrasound. Here, from a microbiological perspective, it can be criticized that although the application of ultrasound is at first glance

a readily quantifiable method, as it can (nowadays) be adjusted relatively accurately depending on the device, it has no counterpart of naturally occurring factors that break down the soil structure with the same force as emanates by ultrasound. The mode of operation of applying ultrasound to separate soil particles, namely the generation and utilization of cavitation, can be classified as highly invasive and distorting and literally blasting the natural conditions, especially at a microbial scale.

The methodological criticism of the aggregate hierarchy model due to the questionable application of ultrasound for microbiological questions is also part of the next points of critique. These refer more specifically to the fractionation method used in this study. This method includes several steps of more or less invasive impacts on the soil structure (sampling, supersaturation with water, application of ultrasound). The massive use of water, which is a characteristic of the wet fractionation method used in this study, results in supersaturation of the soil with water. It is also conceivable that the application of water could lead to unintended detachment and carryover of bacterial cells, especially if the supersaturation with water is accompanied by the application of ultrasound. However, I was able to exclude the presence of significant carryover of bacterial cells in the context of this study by performing appropriate testing (Chapter II). Nevertheless, Bach et al. (2018) demonstrated that wetting soil samples during wet fractionation alters the microbial community in aggregate structures. In addition, Wu et al. (2014) were able to show that bacterial adhesion to surfaces depends on the respective growth phase, so it is also possible that bacteria alter their growth phase due to the influence of abiotic factors, making them harder or easier to detach from surfaces. Of course, the stated goal of these methods is to cause disruption of the soil, but again it is important to be aware of the spatial scale and the effects of the forces acting on the bacterial community within it. Nunes et al. (2017) demonstrated the effect that an environmental impact that is unnatural in its nature and intensity can have on the soil bacterial community, observing a drastic change in bacterial diversity as well as in the structure of the bacterial community. Here, the effect on the bacterial community was analyzed immediately after the impact. Furthermore, a study based on this experiment showed that the profiteers of the previous selection pressure continued to dominate the bacterial community for about 10 days. Not until 29 days a re-stabilization was observed, as a result of which the bacterial community evolved again in the direction of the bacterial community as it had prevailed before the disturbance

(Jurburg et al., 2017). These results thus demonstrate both the magnitude to which macroscopic interventions can disrupt the microscopic system and also the importance, should such invasive procedures be necessary, of preserving the bacterial community as promptly as possible. From there, in future analyses of the microbial community of soil systems that perform fraction size separation, it is desirable to use less invasive methods that take more account of micron-scale fragility.

In this context, dry-fractionation approaches can be a valuable alternative. The evaluation of a new dry fractionation method, in which I was involved in terms of microbiology and which also examined samples analyzed in this study could show that the application of a new fractionation method in direct comparison to wet fractionation resulted in a higher bacterial alpha diversity as well as significant differences in the composition of the bacterial community (Chapter VII – Appendix A) (Felde et al., 2020). This demonstrates that the type of fractionation has a significant impact on the microbiology. The new fractionation method did not involve the application of ultrasound and the use of water for separation, but only the application of mechanical forces. Since the influence of ultrasound and large amounts of water is questionable as discussed above, this new fractionation method can be considered as a valuable alternative for microbial community analysis in future studies. Differences between different approaches of fractionation are also well known in the literature (Bach and Hofmockel, 2014), which again is not surprising if one makes oneself aware of the invasive nature of fractionation methods. Thus, it should always be considered which aspect and which question is addressed when deciding for the most appropriate fractionation method. Bach and Hofmockel (2014), for example, recommend that the use of dry fractionation is better suited to analyze differences of the bacterial community structure, whereas wet fractionation, for its part, is better suited to assess effects on bacterial activity as activity is better maintained in wet samples.

To study the bacterial colonization at the aggregate scale, it would also be opportune to perform a manual fractionation. With the help of a microscope with low magnification power and fine mechanical tools, soil autopsy could be performed and soil aggregates could be specifically and precisely separated from each other. Although this method is labor intensive, it would be minimally invasive. In addition, adjacent aggregate structures could be cataloged in this manner to maintain reference to spatiality for analysis. This type of sample preparation is very well conceivable in particular for microscopic analyses and was, to some extent, actually

applied for the microscopic analyses presented here (Chapter IV), where aggregates were picked from non-fractionated soil samples (but without cataloguing them). Microscopy requires only low sample input, e.g. only individual aggregates. Such as minimal invasive aggregate collection is also conceivable for molecular biological analysis, because very small sample volumes are sufficient when improved methods are used. For example, Probandt et al. (2018) showed that it is possible to analyze the microbial community on individual sand grains. Similarly, Szoboszlay and Tebbe (2021) succeeded in analyzing the bacterial community structure in different sample volumes of 250 - 1 mg and were thus able to investigate individual macroaggregates. Moreover, to go even a step further, it would be worthwhile in future studies to perform the analysis of bacterial colonization of aggregates at the single cell level (Gawad et al., 2016). To date, there have not been many approaches to single cell sequencing of bacteria from soil samples, yet this technique is applicable to soil samples (Nishikawa et al., 2020). In the context of soil aggregates, this approach is particularly interesting as it allows for a better understanding of the function of individual cells in their microenvironment and their ecophysiology, and especially valuable information on non-culturable bacteria can be obtained (Gawad et al., 2016; Hatzenpichler et al., 2020).

When applying the aggregate hierarchy model, the further question arises from a microbiological point of view as to what extent the size classes defined by this model are of relevance for bacterial communities. The range of aggregate size classes investigated in this study corresponds to a factor of 400. Again, if one is aware of the range of influence of environmental factors on bacteria, then the question arises whether the choice of size classes is not too artificial for the consideration of bacterial communities. In this study, for example, it was shown that there is no clear demarcation of the bacterial community structure at the predefined size limit of 250 µm (Chapter II), which separates micro- and macroaggregates. In contrast, a tendency for differences was rather seen within the smallest aggregate fractions. These results are also consistent with the findings of previous studies, which also reported the largest differences in the smallest aggregate fraction, which in turn is closest to the bacterial sphere of impact, whereas differences between micro- and macroaggregates were less evident (e.g. Fox et al., 2018; Trivedi et al., 2015).

In conclusion, the aggregate hierarchy model developed by Tisdall and Oades remains a valuable contribution to the conceptualization of the understanding of soil structure. However, in

this context, from a microbiological point of view, it is important to keep in mind that some of the methods that underlie this model may have a negative impact on the ambition to observe the bacterial community as undisturbed as possible. As described in the previous section (V 2.1.2), it is important to consider the spatial scale and the effect of particular methods on it. Therefore, in order to ensure the preservation of the naturally occurring bacterial community, it is important to identify and select the best method for the respective question to answer. For the analysis of the composition of the bacterial community, the above-mentioned dry fractionation is a promising approach, which, in any case, still requires more detailed testing. However, this method is a useful extension of the toolbox for the investigation of soil aggregates, which can help to better understand the microbiology of aggregate structures and to shed further light on the aggregate hierarchy model from a microbiological perspective as well.

2.2 Microecology and macroecology

During the authoring of this thesis and during the evaluation of the results of this study, I noticed analogies and partly correlations between observations in the microscopic realm and observations in the macroscopic realm time and again. In the following I want to create an appreciation for connecting microscopic as well as macroscopic observations and to learn from insights of both realms and in the best case even to find similarities and to draw from this an adapted gain of knowledge for the respective other scale.

When talking about microecology in the following, this means the microbial ecology as well as the ecology of microhabitats, which includes not only the influence and impact of bacteria but also the influence of other microorganisms, as well as abiotic factors acting at a micrometer scale and thus to be understood in analogy to macroecology. Macroecology, in turn, refers to classical ecology in general and landscape ecology in particular, which takes into account organisms that can be perceived by the naked eye, thus corresponding to the anthropocentric scale.

2.2.1 Soil aggregates as a paradigm of hierarchy theory

There are a variety of approaches and theories that try to explain complex systems and make them more comprehensible. One of these theories is the hierarchy theory, which goes back to the Nobel Prize winner Herbert Alexander Simon (Simon, 1962, 1969, 1977, 1991; Simon and Ando, 1961). Hierarchy theory thereby has a universal claim (Simon, 1962) and was developed from a cross-disciplinary perspective that takes into account economics, psychology,

management, biology, and mathematics. In particular, ecology was significantly influenced by hierarchy theory (Allen and Starr, 2017; Bissonette, 1997; O'Neill, 1986) and led to the formation of new ecological thinking patterns, which were primarily pattern-process-scale oriented.

Basically, according to the hierarchy theory, a hierarchy is to be assumed if a system is present, which is structured in layers or levels, which in turn show asymmetrical relations between each other. In this context, Simon (1962) defines hierarchy as "a system that is composed of interrelated subsystems, each of the latter being, in turn, hierarchic in structure until we reach some lowest level of elementary subsystem". In this context, the determination of the last level of elementary components is subject to a certain degree of arbitrariness. The subsystems or individual components, of which the individual levels of the hierarchy are composed of, are called holons (Koestler, 1967). Thereby a holon is to be regarded as a whole and as a unity, if it is viewed on its own level or from the levels below, but on the other hand also merely as a part of the whole, if one looks at it from a higher level. This definition of a hierarchy can be nicely applied to the aggregate structure of soil systems and is also reflected in the aggregate hierarchy model of Tisdall and Oades (1982), where holons represent the individual aggregate size classes and the building blocks of aggregates. Curiously, however, there are no references to hierarchy theory in Tisdall and Oades, nor does the subsequent literature link these two theories. However, it should also be noted here that Tisdall and Oades (1982) never titled their model itself as an aggregate hierarchy model; the notion of a hierarchy is not mentioned once. Where the denomination of this model came from remains unknown and untraceable. Nevertheless, one cannot help but notice a very close relationship between the two theories.
Furthermore, the interfaces between the different levels and holons, as well as in soil aggregates, are called surfaces, described as the places with the highest variation of interactions (Ahl and Allen, 1996; Allen and Starr, 2017). In this context, surfaces are considered to have a filtering effect, as they are assumed to process and interact with the flows of matter that are passing them. This concept also translates well to soil systems and soil aggregates, as it is assumed that the vast majority of bacteria reside on the surfaces of aggregate structures (Chenu and Cosentino, 2011; Vos et al., 2013) feeding on or metabolizing the nutrients available there, but also the nutrients that diffuse to the cells via the soil solution.

For the hierarchy theory there are no rigid criteria that define it firmly, which on the one hand can be seen as a point of criticism, on the other hand the universal claim of this theory can be implemented in this way. Thus, hierarchies can be divided into different types according to different criteria. For example, with respect to dimension, structural and functional hierarchies or spatial and non-spatial hierarchies as well as ecological hierarchies can be distinguished. Salthe (1991) distinguishes between scalar hierarchies and specification hierarchies. The former are organized spatio-temporally, whereas the latter are nested and combine different levels of development. Examples are levels of organization like atom <-> molecule <-> cell <-> organ <-> organism <-> population or respectively chemistry <-> biology <-> physics. An important aspect in relation to hierarchy theory is the distinction between nested and non-nested hierarchies (Ahl and Allen, 1996; Allen and Starr, 2017). Nested hierarchies are characterized by the fact that the higher levels or organizational structures at least contain lower levels or are even entirely composed of them. Thus, the renowned ecologist MacArthur (1972) also describes the nested nature of the natural environment and states that real living worlds are based on a hierarchical structure. This is also true for the composition as well as for the structure of soil aggregates in particular and soils in general.

A key characteristic of hierarchy theory is the order of the different hierarchy levels. Allen et al. (2009) identify five general principles that characterize the order in hierarchical ecological systems:

1. Higher levels have lower process rates than lower levels
2. Higher levels influence lower levels
3. Higher levels form an encompassing framework around lower levels
4. Higher levels have weaker binding strengths between the different holons and thus have less integrity than lower levels
5. When nested hierarchies occur, higher levels contain lower levels or consist entirely of them

Another characteristic of hierarchical systems is that they have a horizontal and a vertical structure (Ahl and Allen, 1996). The vertical structure, i.e. the relationship between the levels, is characterized by an asymmetrical connection. Here, the number of vertical levels represents the depth of the hierarchy, and again, the deeper a hierarchy, the more detailed and complex

the overall hierarchical structure. The horizontal structure, in turn, results from the relationships between the different holons and is characterized by a symmetry in the strength of the interactions at the shared level. Here, the intraspecific interactions between the components of a holon are significantly stronger and more frequent than the interspecific interactions between different holons. Thereby, the more pronounced and frequent intraspecific interactions also form the basis for the integrity of the individual holon. These characteristics of hierarchical systems can again be applied to aggregate structures. Thus, the different aggregate size classes represent the vertical structure, which are distinguished by different stabilities and mechanisms holding the respective aggregate structures together. The respective aggregates of an aggregate size class in turn represent the horizontal structure.

Furthermore, Simon (1977) assumes that the individual components are only loosely coupled with each other in the horizontal as well as in the vertical direction. This loose linkage allows separation between the individual levels in the vertical direction and independence of the individual holons in the horizontal direction, which allows them to exist in a partially self-sufficient manner. This view also matches the idea that aggregate structures in general and microaggregates in particular form their own self-contained microhabitats.
This further core feature of hierarchical and complex systems is what Simon (1962, 1977) calls "near-decomposability", which in summary states that the frequency of interaction within components of the same level is much higher than the rates of interaction between components. The consequence of this is that in order to adequately describe a hierarchical system, it is necessary to choose a focal level and to define slow processes from levels above as constants and fast processes on levels beneath as means. By this approach it is possible to "scale" relevant levels of superordinate and subordinate levels and thus to obtain a higher degree of simplification, which, however, allows a better comprehension (Simon, 1962, 1977, 1996). The concept of near-decomposability was further extended by Salthe (1985), who developed the concept of the basic triadic structure. This concept states that in order to provide a sufficient explanation for the description and explanation of processes at the focal level, it is necessary to take into account the level above as well as the level below. This is based on the fact that the focus level is primarily based on processes of the subordinate level and at the same time is influenced by the surrounding conditions, which the superordinate level provides.

Another interesting approach, which fits well with the nested hierarchical structure of soil aggregates, is one of fractal geometry and self-similarity (Brown et al., 2002; Mandelbrot, 1983; Milne, 1988). For example, Wiens and Milne (1989) showed here that there was a correlation to the fractal geometry of landscape structures at the macroecological scale. Because of the repetitive patterns and structure of aggregates, it would therefore be very interesting to test such a correlation also at the level of aggregate structures in future studies.

In summary, many aspects of hierarchy theory fit well with the aggregate hierarchy model and it has thus the potential to enhance the view and explanation of soil aggregate structures in multiple ways. Regardless, I have not encountered any linkage between these two theories in the literature up to this point. The application of universal hierarchy theory in general and its ecological interpretation in particular is an example of the immense potential of adapting macroscopic theory to microscopic systems. Future analyses of the microbial ecology of soil systems should thus give more attention and consideration to the concepts of hierarchy theory and examine whether adapting the concepts of this theory can contribute to a better understanding of aggregate structures and soil systems.

2.2.2 Conflating macro- and microecology

In addition to a universal approach like hierarchy theory, there are also a variety of specific theories and concepts that describe the functionality of macroecological communities and systems. In the following, a few examples will give a brief impression of the potential of adapting proven macroecological concepts.

Compared to microbial ecology, macroecology has a long tradition of widely observed and proven concepts that explain the community functioning of different organisms in interaction with their surrounding environment. In this context, the concepts of macroecology successfully describe the colonization of landscapes and landscape structures, which are often also characterized by high inter- and intra-heterogeneity. Therefore, great potential to gain a better understanding of bacterial colonization of soil systems emanates from the adaptation of verified concepts of landscape ecology. In this regard, Turner (2005) defines landscapes as scale-independent arrangements of patterns that are interrelated by the organisms that inhabit them and ecological processes that occur. Therefore, it is reasonable to examine verified concepts of macroecology for a possible adaptation at the soil microbiological scale. For example, Bardgett and van der Putten (2014) state that the diversity of microhabitats found in

the 3D structure of a soil profile is comparable to that of an entire ecosystem. This statement can be further broken down at scale in that even the 3D structure of a single aggregate has the potential to harbor the habitat richness of an entire ecosystem. However, it must be emphasized that this is always only an adaptation and never a 1:1 application of existing concepts.

As noted in a detailed review by Mony et al. (2020), the adaptation of landscape ecology principles in microbiology is still in its infancy. The authors find many examples that describe microorganisms in abiotic landscapes and also demonstrate how variably applicable the concepts of landscape ecology are. For example, the authors also define biotic systems, i.e. macroscopic organisms, as biological landscapes. In this context, the macroorganisms as a whole are understood as a biological landscape and their organs in turn as parts of this entire landscape. The motivation for this view can often be found in the explanation of diseases and the attempt to understand how pathogens spread. Accordingly, landscape ecology concepts have the potential to explain processes and mechanisms of soil systems, particularly in relation to aggregate structures.

To specify this, there are various conceptual landscape composition models in landscape ecology, which offer an approach that is of interest when thinking about and classifying aggregate structures. The first two models assume that landscapes consist of separate areas that can be differentiated from each other (patch-matrix (Turner, 1989) as well as the mosaic-landscape model (Wiens, 1995)). The patch-matrix model is an early and very simple model, which only differentiates between habitable and non-habitable regions, which are clearly distinguished from each other. It is based on the assumption that the regions that are not suitable as habitat also have no further influence on the composition of the colonizing community. The mosaic-landscape model, developed shortly thereafter, can be seen as a further development of the first model, taking more account of the heterogeneity of landscapes. Here, a distinction is now made between different types of habitats, which can be inhabited with greater or lesser success, but can still be clearly distinguished from one another. In contrast to the previous two models, the third and most recent model, the landscape-continuum model (Cushman et al., 2010; Fischer and Lindenmayer, 2007) assumes that landscapes form an ecological continuum. Here, different environmental variables can have varying gradients and intensities, and there is no longer a clear demarcation between the different habitats. Boundaries are blurred here and the different variables in the individual habitats are no longer static.

These models have been developed consecutively in a short time sequence, thus also reflecting the awareness and exploitation of increasingly more complex systems. The increasing complexity goes hand in hand with the ever more precise and ever more accurately scalable analysis methods, which make the registration of increased complexity possible in the first place. Soil aggregates are the perfect example of a highly complex system in this regard, and there are good possibilities for adapting the models described above to aggregate structures. However, rather than applying a single model to aggregates, an application of both the mosaic-landscape model and the continuum model can be justified by including the temporal dimension. The microscopic results obtained in this study (Chapter IV) suggest the validity of the mosaic-landscape model when considering the heterogeneous, yet visually distinct, areas of aggregate structures and bacterial colonization, especially when viewed in conjunction with the results of Lehndorff et al. (2021). However, considering that soils are highly dynamic systems that are influenced by macroclimatic conditions, such as precipitation and periods of drought, it is also easy to imagine that during periods of increased soil moisture, a continuous temporary system interconnected by water flow emerges (Manzoni and Katul, 2014). During these phases, gradients form and areas isolated from each other in the dry state become interconnected and allow exchange, which again is an approximation of the continuum model. The importance of temporary factors and the influence of microorganisms and aggregate structures was also shown in this study (Chapter III).

Furthermore, there are a variety of other principles in macroecology, which are reflected in microecology. For example, it is an established macroecological observation that there is a positive correlation between the size of an area and the number of species present, which applies to both animals and plants. In this context, an increase in area is also often linked to an increase in environmental heterogeneity, with this heterogeneity in turn often being the most common explanation for observed taxa-area patterns (Rosenzweig, 2010). Such a relationship can again be surmised from the results of this study. Considering the observations of the microscopic analyses (Chapter IV) in combination with the results of the analyses by Lehndorff et al. (2021), it is reasonable to assume that increased bacterial diversity exists with respect to the immense environmental heterogeneity as well as area of microaggregate structures (Chapter V, 2.1.2). For example, Storch (2016) found for species-area relationships that they can be described as having a three-phase shape. Here, this has an S-shape, where there is a steep increase at small scales, a decrease is observed again at medium scales, and a steep

increase is observed again when continental scales are considered. This also fits well with the observations on the smallest scale, which were made in the context of this study. Furthermore, several other studies have also been able to establish that there is a taxa-area relationship in the field of microorganisms and thus it appears to be a universally valid law (Adler et al., 2005; Bell et al., 2005; Glassman et al., 2017; Horner-Devine et al., 2004). Although, as noted at the beginning, no direct transfer of this regularity to the bacterial world is possible, since a direct comparison of bacteria with macroorganisms reveals a weaker correlation in bacteria than in macroorganisms. For example, the accumulation of taxa with increasing area as well as the decrease in similarity between bacterial communities with increasing geographic distance tends to be lower for microorganisms than has been observed for plants and animals (Green et al., 2004; Hillebrand et al., 2001; Horner-Devine et al., 2004; Zhou et al., 2008). One reason for this could be a too voluminous sample used for bacterial community analysis. Therefore, it would be interesting to perform such analyses on the level of aggregate fractions or even single aggregates in the future.

Another concept that can be observed in both the macroscopic and microscopic worlds is that of predator-prey relationships. In both macroecology and microecology, the question arises as to the causes and consequences of spatial heterogeneity and how this in turn affects the organisms in place, and also how the interactions of those organisms in turn affect the environment around them. For example, Hol et al. (2016) were able to show how spatial heterogeneity affects organisms at a microscopic scale. Here, a heterogeneous landscape compared to a uniform landscape had significant effects on microbial hunter-prey relationships. While the prey was hunted to near extinction in the uniform landscape, it managed to survive in the heterogeneous landscape. By far more heterogeneous landscapes are also found in aggregate structures. Similar observations and correlations exist in the macroscopic world. Here it has also been shown that a heterogeneous landscape can have a great influence on predator-prey relationships (e.g. Ellner et al., 2001; Holyoak and Lawler, 1996; Sutcliffe et al., 1997).

In addition, it is to be noted that there are fundamental commonalities already at the level of the basic mechanisms for the emergence and formation of communities of living beings. These are selection, drift, speciation/mutation and dispersal or its limitation. These partly interacting basic mechanisms can be observed in communities of microorganisms (Hanson et al., 2012) as well as in communities of macroorganisms (Vellend, 2010).

The above examples serve to illustrate that there is great potential to review macroecologically verified findings for their adaptation in microbial ecology and thus to create a general awareness that there are basic principles and patterns that are valid regardless of scale, although not 1:1 but in adaptation. It is in itself logical, although not always self-evident, not to lose sight of the big picture even when considering the smallest systems and interrelationships.

2.2.3 Discrepancies between the analysis of the micro and the macro scale

Since the beginning of research on soil microbiology, a vast amount of experimental data on macroscopic soil parameters has been collected. Most of these are analyses of bulk soil samples for the determination of macroscopic soil parameters (e.g. pH, texture or organic matter content), which are derived based on gigantic sample volumes from a microbiological point of view and thus with disproportionately coarse spatial resolution. However, based on the results of recent research, which increasingly deals with the relative spatial scale and the impact of parameters on it (Chapter V, 2.1.2), it is becoming increasingly clear that it is necessary to ensure a finer resolution in the collection of soil parameters as well as to establish a standard collection of new macroscopic soil parameters, which better reflect the spatial heterogeneity. For example, it would be interesting to include pore structure in future analyses as Ruamps et al. (2011) showed that the microbial community structure depends on the respective pore size. Terrat et al. (2017) demonstrated impressively in their study how insufficient the previous result of macroscopic parameters is for the explanation of microscopic phenomena. In a comparison of samples from over 2000 sites distributed throughout France, the authors were able to relate an observed variance in bacterial diversity to the standard recorded soil parameters in less than half of the cases. This observation can be partially confirmed by the findings obtained during this study. The different parameters that were included here (aggregate size, clay content) provided only a partial explanation for the observed variability of the bacterial community (Chapter II), which in turn implies that there must be other factors responsible for the variability of the bacterial community. An indication of this is provided by the microscopic analyses of this study (Chapter IV), which, in concert with the results of Lehndorff et al. (2021), suggest that a possible explanation of microbial diversity lies at the microscale. Thus, it is conceivable that standard soil parameters collected to date may well be important, but that the scale at which they are collected is too coarse. It should also be noted that there are two core components of scales. These are the grain and the extent (Wiens, 1995). These two factors

must be taken into account when considering scales. Compared to macroecology, scales and the dependence of various mechanisms on them have received little attention in microbial ecology (Ladau and Eloe-Fadrosh, 2019).

As already mentioned several times in the previous chapters, a direct application of macroscopic principles on a microscopic scale is not possible without adaptation. This is based on the fact that there are, of course, significant differences in detail in both worlds. For example, microorganisms are characterized by their small size and short generation time, which in turn affects their ability to react to changes in their immediate environment. These reactions occur in response to circumstances at very small spatial scales and over several generations, which particularly emphasizes the relevance of evolutionary processes. As a result, the sphere of impact for microorganisms has a higher degree of complexity than it does for macroorganisms, which in turn can be explained by the partially nested temporal and spatial scales (Mony et al., 2020).

2.2.4 Microaggregates are landscapes

Considering the results of this study as well as the overall impression that emerged in the discussion, it is obvious to regard microaggregates as landscapes from a bacterial perspective. This conclusion is permissible if one considers what characteristics constitute a landscape in macroscopic and anthropocentric terms, and how microaggregate structures arise from a bacterial perspective. While Carl Troll (Troll, 1950) was still describing landscapes as areas where humans interact with the environment on a kilometer-scale (an extremely anthropocentric perspective), Wiens and Milne (1989) were already defining landscapes more generally as a template (independent of scale) on which spatial patterns influence ecological processes. In doing so, the authors traced their assumptions from a consideration of the perspective of beetles and their conception of their environment as a landscape. Continuing, Mony et al. (2020) demonstrate in their review the flexibility that landscape ecology, and thus the definition of landscapes, offers in transferring concepts to systems that cannot be classified as landscapes when initially regarded.

In this study, an even smaller scale and higher spatial resolution approach could be pursued. In doing so, it was possible to approach the sphere of impact of individual bacteria and find evidence that microorganisms likewise conceive the architecture and structure of soil aggregates as landscape structures from their perspective and colonize them in a manner similar to

how macroorganisms colonize the landscape structures that surround them. There are analogies in the processes of the micro and macro perspective which suggest such a conclusion.

Thus, an overarching goal of this study is to encourage future soil microbiological studies that investigate the community of microorganisms to consider and internalize this line of thinking in order to exploit potential interdisciplinary synergies to gain a better basic understanding.

VI
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VII
Appendix A

Wet sieving versus dry crushing: Soil microaggregates reveal different physical structure, bacterial diversity and organic matter composition in a clay gradient

Modified on the basis of

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1 Abstract

Soil microaggregates contain particles of different sizes, which may affect their potential to store organic carbon (OC). A variety of methods can be used to isolate microaggregates from the larger soil structures among which wet sieving approaches are widely employed. We developed a novel dry crushing method that isolates microaggregates along failure planes due to mechanical stresses rather than hydraulic pressures and compared the mechanical stability, OC contents and microbial community composition between dry-crushed and wet-sieved samples with contrasting clay contents. Dry-crushed samples exhibited a higher stability and bacterial diversity compared to wet-sieved samples. As a result, the dry-crushed microaggregates had different size distributions when analyzed dry and after wetting. In the dry state, dry-crushed microaggregates were larger and contained more sand-sized primary particles within the aggregate structures. The wetting of dry-crushed aggregates caused a disintegration of larger microaggregates and sand-sized primary particles into smaller microaggregates that contained finer particles. In the soils with lower clay contents, the diameter of dry-crushed microaggregates was 40 µm larger due to more sand-sized primary particles remaining within the aggregates. Depending on how much volume in microaggregates is occupied by large primary particles, the OC concentration increased in the soil with higher clay content. Wet-sieved size fractions also showed a similar pattern of OC distribution, whereas more primary particles were observed outside of aggregates. Wet sieving approaches disperse the soil into OC-rich aggregates and might be preferable if OC dynamics are investigated. Differences in bacterial community composition in dependence on clay content were more pronounced in dry-crushed microaggregates. If intact aggregate architectures are of interest for the isolation of soil structural units, the presented dry crushing method might provide an advantageous alternative that also better preserves bacterial diversity.

2 Introduction

The physical structure of soil is of primary importance for most of its functions and is often expressed as the degree of aggregation (Bronick and Lal, 2005; Dexter, 1988). The aggregation of soil components is a critical process that regulates C sequestration (Six et al., 2000b) and determines physical support, hydraulic properties, aeration and the accessibility of biogeochemical interfaces, which are essential for nutrient exchange and microbial processes in soils (Bronick and Lal, 2005; Totsche et al., 2010). Soil microaggregates with a size smaller than 250 µm have been proposed as stable compound soil structures (cf. Totsche et al., 2018). Microaggregates are themselves comprised of various smaller building units, such as coarse and fine mineral primary particles, phyllosilicates, Fe and Al (hydr)oxides and diverse organic materials (Totsche et al., 2018). In contrast to macroaggregates, microaggregates are more stable and have longer turnover times, which is why aggregate size plays an important role in long-term C storage in soils (Angers et al., 1997; Six et al., 2000; Trivedi et al., 2017).

Under field conditions, various mechanisms cause soil (micro)aggregates to break apart. These can be either mechanical forces, originating from field traffic and tillage implements, or radial pressures from roots and earthworms causing point loads and shear failure (Horn and Peth, 2012; Ruiz et al., 2017). Mechanical forces are also introduced during raindrop impact (Fu et al., 2017) or differential swelling of clay minerals; however, the resulting disintegration of aggregates is a mixture of mechanical and hydraulic stresses (pore water pressures). These mechanical forces can create and sustain pre-conditioned failure planes within soil aggregates that may be reactivated when repeatedly exposed to stresses. Further mechanisms for aggregate breakdown can be slaking and dispersion, which occur during wetting and drying of the soil and are related to hydraulic stresses (Le Bissonnais, 1996).

Various approaches are used to break down aggregates. The most common method to isolate microaggregates from bulk soil is wet sieving, which was mostly employed to investigate the stabilization of aggregate structures through organic matter (OM) and OM turnover (Bach et al., 2018; Six et al., 2004). The impact of water immersion on soil aggregates has been shown to depend on the size, structure, shrinking and swelling behavior, wettability of soil components, porosity, and the spatially heterogeneous distribution of these properties within aggregates (Baumgartl and Horn, 1993; Chenu et al., 2000; Kaiser et al., 2015). The super-saturation of aggregates during wet sieving can increase the gas pressure inside aggregates causing their

disruption (Baumgartl and Horn, 1993). Previous studies have shown that water-stable microaggregates are stabilized by various agents such as OM, phyllosilicates, Fe and Al (hydr)oxides and $CaCO_3$ (Amézketa, 1999; Bronick and Lal, 2005; Oades and Waters, 1991; Totsche et al., 2018). In other studies (using glass beads to increase the dispersion energy), it was postulated that microaggregates are formed while being occluded in macroaggregates (Angers et al., 1997; Six et al., 2002). Likewise, sonication of soil suspensions can be used to liberate microaggregates from macroaggregates (Amelung and Zech, 1999).

The dry sieving of soil (field-fresh or after air-drying) is based on the mechanical impact on soil structure during shaking the samples in a sieve tower, either by hand (Bach and Hofmockel, 2014; Blaud et al., 2017) or using a mechanical sieve shaker (Nahidan and Nourbakhsh, 2018; Panettieri et al., 2015). It is used to differentiate microhabitats being associated with aggregates of various size fractions (Bach and Hofmockel, 2014; Blaud et al., 2017) or to gain information about the impact of wind erosion on aggregates (Chepil, 1962). Apart from dry sieving to a certain size (typically 8 or 4 mm), the aggregates in the aforementioned studies are usually not treated by dispersion agents before the dry sieving.

Another approach, which is able to liberate more microaggregates that are trapped within macroaggregates (Six et al., 2000), can be to mechanically crush the macroaggregates down to microaggregate sizes, using an uniaxial crushing procedure. Uniaxial crushing enables the breaking down of dry aggregates along “natural planes of mechanical weakness” (Kristiansen et al., 2006) that are likely to fail under mechanical forces in the field as well. If intact aggregate structures are of interest, for example their internal spatial architecture creating a habitat for microorganisms or storage site for OM, dry separation protocols like dry crushing have the advantage that they avoid potential structural artifacts that are likely to occur during water immersion and subsequent oven drying (Kaiser et al., 2015; Panettieri et al., 2015; Siebers et al., 2018). Since different isolation methods are based on different breakdown mechanisms, differences between the isolated size fractions can be expected (Six and Paustian, 2014). In addition to the existing studies that compared mainly microbial parameters of wet and dry-sieved aggregates (Bach and Hofmockel, 2014; Blaud et al., 2017), more studies are needed to better understand how isolation methods like wet sieving and dry crushing affect the structures, size distribution and physicochemical, mechanical and biological properties of soil aggregates.

During aggregation, primary particles are combined to form larger soil structures. The individual contribution of soil particles to properties of the bulk soil therefore depends on its arrangement within the aggregate architecture. Especially fine mineral soil particles can affect the surface adsorption of OM and formation of microaggregates through organo-mineral associations (Baldock and Skjemstad, 2000; Wagner et al., 2007a). Clay content is positively correlated with aggregate tensile strength (Imhoff et al., 2002; Kavdir et al., 2004; Kay and Dexter, 1992) and was found to stabilize larger aggregate structures that drive the arrangement and the distribution of OM in aggregate fractions (Dexter et al., 2008; Krause et al., 2018; Schjønning et al., 2012; Schweizer et al., 2019). Because larger primary particles do not play a dominant role for OM storage inside soil aggregates, our current conception of aggregate structures has mostly focused on structures that are comprised of many fine particles. Aggregates with a coarse texture component were, hitherto, not at the center of scientific attention. One of the first studies looking at the functional role of sand grains inside microaggregates by Paradiś et al. (2017) described sandy microaggregates with a solid sand grain nucleus that is surrounded by mineral fines. They conceptualized a model where water menisci forces draw fine soil constituents towards the solid surface of larger particles after multiple wetting-drying events. This may also lead to the coalescence of multiple sand grains within aggregates (Ghezzehei and Or, 2000). Since higher clay contents are mostly correlated with lower sand contents, many aggregate properties are likely to be modified by soil texture (Schweizer et al., 2019; Wagner et al., 2007b). Detailed size analyses of aggregates and the particles they are composed of are needed to reveal the size composition and the role of the individual particles within microaggregates.

Soil aggregates are important habitats for microorganisms (Blaud et al., 2017) depending on various aggregate properties, such as their size and structure. Higher clay contents correlate positively with microbial biomass (Wei et al., 2014), which can lead to larger and more stable aggregates (Wang et al., 2017). Soil aggregation increases microhabitat heterogeneity and thus bacterial diversity in soils (Davinic et al., 2012; Nunan et al., 2017) Different microaggregate sizes are likely to show differences between their pore systems, leading to differences in the accessibility of OM sources, which strongly affects microbial activity (Bimüller et al., 2016; Ebrahimi and Or, 2018; Gupta and Germida, 2015). Similarly, differences in soil texture are expected to influence microbial activity depending on how they affect OM accessibility. The rewetting of dried samples during wet sieving was shown to affect the characterization of

microbial communities in soil aggregates (Bach et al., 2018). For this reason, Bach and Hofmockel (2014) have stated that wet sieving should be used to evaluate long-term changes in microbial activity and OM, while dry separation without rewetting would allow capturing short-term (inter-annual) dynamics of soil microbial activity too. For all these reasons, the characterization of the microbial community provides important information to compare the structure, size and other properties of aggregates as influenced by the isolation method.

Here, we compared the mechanical stability of individual microaggregates and OM concentrations as well as microbial community composition between wet fractionated and dry-crushed size fractions. The dry-crushed fractions were further characterized regarding their size distributions and organic matter content. To investigate the effect of soil texture on dry-crushed aggregates, we used samples from a clay content gradient of 19-34 %. We developed a novel method to isolate microaggregates from macroaggregates by mechanically crushing them with a loading frame prior to dry tap sieving. The particle and aggregate size distribution of the dry-crushed size fractions was precisely analyzed using dynamic image analysis. In addition to the differentiation into several of size fractions by sieving, the dynamic image analysis allowed detecting size changes at a resolution of several µm for the whole microaggregate scale < 250 µm.

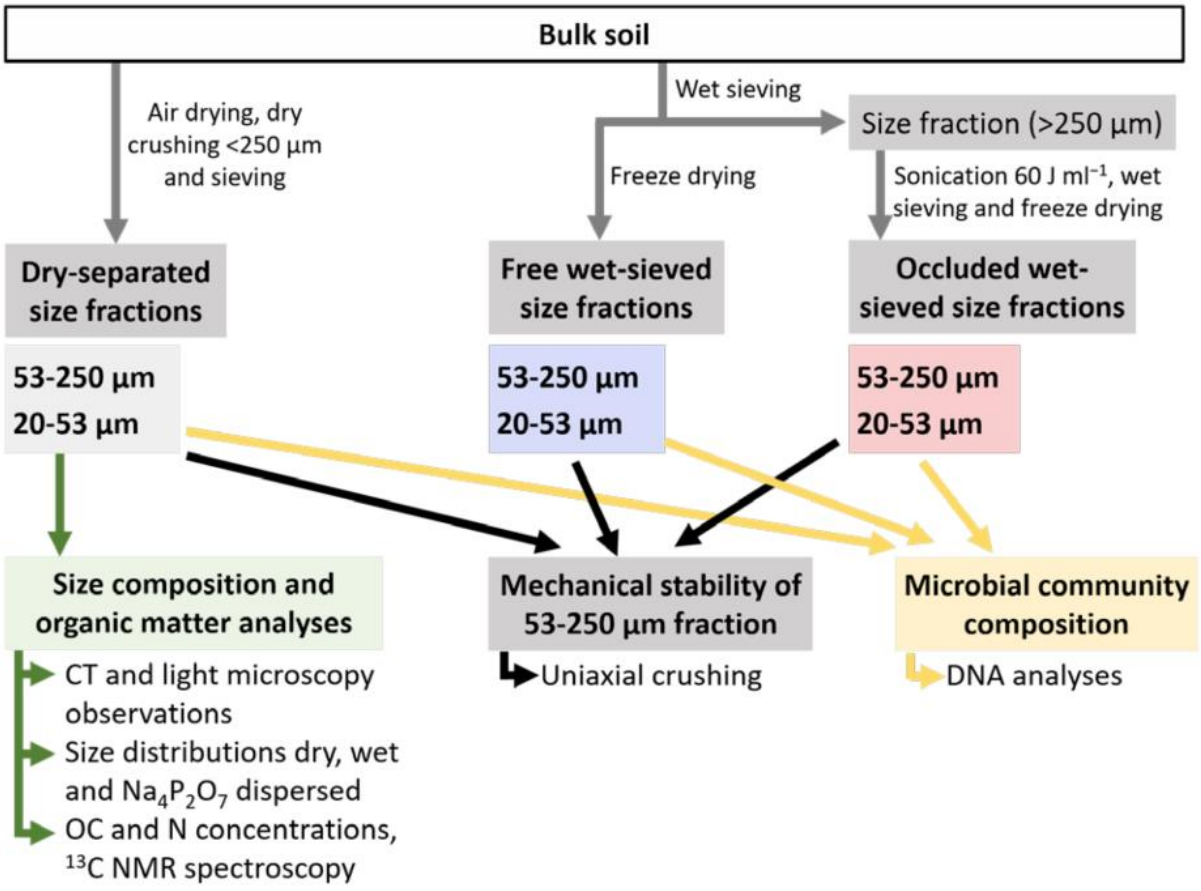
We hypothesized that dry crushing isolates aggregate size fractions shaped by different failure planes and yields different properties compared with wet-sieved aggregate size fractions (with and without sonication). In this comparison, dry-crushed size fractions were expected to have a lower mechanical stability, retain less OM and exhibit differences in microbial community composition compared to wet-sieved ones (both with and without sonication). For the investigation of the soil texture gradient, we hypothesized that a higher clay content would increase the mechanical stability, mean aggregate size, OM content and bacterial diversity of microaggregates.

3 Material and Methods

3.1 Study site

We studied a soil toposequence at an agricultural research station in Scheyern (Germany) that was previously described by Schröder et al. (2002). The mean annual temperature is 7.4 °C with an average annual precipitation of 803 mm at an altitude of approximately 470 m above

sea level. Soils developed on Miocene Upper Freshwater Molasse covered by several meters of Quaternary loess and were classified according to WRB (FAO, 2015) as Cambisol (Schröder et al., 2002). Soils were managed by reduced tillage (harrowing and chiseling). Samples were taken at clay contents of 19 %, 24 % and 34 % (n = 5 each). More detailed information on the sampling of the toposequence can be found in Krause et al. (2018). Briefly, the top 5-20 cm were sampled in late 2015 in a field-fresh state (water content approximately 16 weight % or 21 vol. %), sieved to < 8 mm, and stored at 4 °C until further processing. An overview of the complete sample analysis process is given in Suppl. Fig. VII-1.



Suppl. Fig. VII-1: Overview of isolation methods and analyses

3.2 Fractionation by dry crushing

We produced microaggregates with diameters of < 250 µm by crushing macroaggregates under uniaxial compression in a mechanical loading frame (Zwick Roell AllroundLine, Zwick Roell, Ulm, Germany). Briefly, we used air-dried large macroaggregates with a diameter of < 2 mm. We weighed approximately 5 g and measured the matric potential in a WP4C dew point potentiometer (Decagon devices, Pullman, USA) to make sure the sample was completely dry. All samples had pF values of > 6. Next, the samples were crushed under the loading frame to

separate differently sized microaggregates, which are constituents of the macroaggregates. For this, the distance between loading frame table and piston was slowly reduced from 3 mm to 250 µm at a constant speed of 250 µm min^{-1}. On average, the samples contained 33.6 %, 20.0 % and 10.2 % sand grains of > 250 µm in the soils with 19, 24, and 34 % clay. These grains might have been crushed during the dry separation, which would result in sand grain fragments in the size fraction. To minimize this, the crushing speed of the loading frame was deliberately chosen to be slow so that the sand grains could reorient themselves during the process (the slow speed was chosen as a result of preliminary experiments). No high-pitched sounds related to the rupture of sand grains or of any sharp peaks in the load-displacement curves that would indicate crushed sand grains were observed (Figure S 1). During light microscopy and tomography scans, we also did not observe any sand grain fragments.

After crushing, we transferred the crushed sample into a modified Casagrande apparatus (Mennerich Geotechnik, Hannover, Germany), consisting of three sieves that rest on a collecting vessel. Here, the sample was exposed to the purely mechanical forces of repeated tap-sieving cycles at a frequency of 2 Hz. Tap sieving was chosen to prevent abrasion of soil material from the aggregate surfaces through circular shaking, which would have biased aggregate fractions. In this process, microaggregates were separated under air-dry conditions into the following size fractions: small microaggregates and primary particles (< 20 µm), medium (20-53 µm) and large ones (53-250 µm) similar to size limits applied in previous studies (Jastrow, 1996; Tisdall and Oades, 1982; Virto et al., 2008). The smallest fraction (< 20 µm) was not analyzed in this study due to the very low amount that could be isolated (Figure S 2). In a preliminary experiment, we determined the number of cycles in the Casagrande apparatus that are required to get a relatively steady aggregate size distribution, which was about 1000 cycles (Figure S 3). Hence, after 1000 cycles, we collected the residue on all three sieves. The crushing and tapping procedure can be seen in the following videos: https://doi.org/10.6084/m9.figshare.10327397.v1 and https://doi.org/10.6084/m9.figshare.10327529.v1.

3.3 Light microscopy and tomography

The size fractions were analyzed with optical microscopy using a Zeiss Axio Imager M2. Explorative computed microtomography scans were done on one dry-crushed microaggregate for each clay content at the CT lab of the University of Kassel with a Zeiss Xradia 520 Versa. For

each of the three scans, we acquired 1,600 projections during a full rotation with 3 s acquisition time at 80 keV and a resulting voxel resolution of 570 nm, 482 nm and 656 nm, respectively.

3.4 Dynamic image analysis of dry-crushed soil size fractions

Size distributions were determined by dynamic image analysis with rear illumination from a pulsed laser light (450 fps) using a QICPIC (Sympatec GmbH, Clausthal-Zellerfeld, Germany) as described previously (Kayser et al., 2019). In the dry state, we analyzed the size fractions (60 mg to 80 mg) with the GRADIS drop tower (50 cm height) using a constant flow of compressed air. The medium size fraction (20-53 µm) was analyzed with the M4 lens (2-682 µm measuring range) and the large size fraction (53-250 µm) with the M6 lens (5-1705 µm measuring range; ISO 13322-1).

To test how the failure planes during dry crushing and wet sieving differ and what the underlying primary particle size distribution was, we submerged the dry-crushed fractions in water and subsequently dispersed them further using $Na_4P_2O_7$. It should be noted that the size analysis was done with the fractions obtained from dry crushing in order to get further information on the size distribution after their dispersion. Detailed information on the particle and microaggregate size distribution of the samples after wet sieving can be found in a previous study by Schweizer et al. (2019), which is used for comparisons in the discussion section.

For this, we determined the size distributions of the dry-crushed fractions after submersion in deionized water and subsequently in $Na_4P_2O_7$ solution. After slow wetting of the size fractions with deionized water for 30 min, the suspensions were measured with a different setup in the same QICPIC machine. A closed pumping cycle using the LIXELL flow cell and SUCELL homogenization unit with a 0.2 mm and 1 mm cuvette allowed to transport the suspension by the optical unit of the machine.

To determine the size distribution and mass contribution of primary particles in the size fractions, we measured the dry-crushed samples after dispersion of 100 mg sample material in 0.1 M $Na_4P_2O_7$ (Kemper and Koch, 1966; Murer et al., 1993). After dispersion, the suspension was transferred to the pump unit and measured as described above.

The data obtained by dynamic imaging were analyzed using the automatic WINDOX software (Sympatec GmbH, Clausthal-Zellerfeld, Germany). By relation to a density of 2.65 g cm^{-3}, the normalized volumetric distribution density q3 was calculated according to the minimum distance of two tangents to the edges of the objects, the so-called Feret diameter (Allen, 1981).

The median diameter was used to compare the distributions of different clay contents statistically. To compute distributions of the whole size range we used class sizes of 6 µm for the medium fraction and 15 µm for the large fraction. Shape criteria (Table S 1) were chosen based on a gallery function within the image analysis algorithm, which enables verifying that a low number of individual odd-shaped objects like root pieces (low circularity) or overlapping particles (high optical density) do not distort the computed size distribution (Kayser et al., 2019). The influence of bubbles and root hairs on the size distributions was minimized by specific shape criteria for both size fractions using the circularity of the objects (Table S 1). The circularity is the ratio of the perimeter of an equivalent circle to the perimeter of the object, which decreases when particles are rounder.

Due to optical limits of the applied camera lenses, some particles might have been too small to be detected by the dynamic image analysis. To relate the volumetric distribution with the actual mass proportions, we corrected the size distributions for particles < 20 µm and < 53 µm by sieving the particles after dispersion with $Na_4P_2O_7$. We observed that, 19.1, 21.2 and 24.8 % of the particles in the soils with 19, 24, and 34 % clay were < 20 µm in the aggregate size fraction 20-53 µm. In the size fraction 53-250 µm, 3.1, 1.5 and 7.1 % of the particles were < 53 µm after dispersion with $Na_4P_2O_7$ in the soils with 19, 24 and 34 % clay. We subtracted the total contribution of the whole size distribution of the respective size fraction accordingly. Both size fractions, 20-53 µm and 53-250 µm, were merged according to their respective mass contribution to the bulk soil and are displayed as one size distribution across the whole size range.

3.5 Carbon and nitrogen analysis and solid state ^{13}C nuclear magnetic resonance spectroscopy

The C and N contents were measured by dry combustion using a Vario EL CN analyzer (Elementar, Langenselbold, Germany). The contributions of the size fractions to the bulk OC were computed according to their mass contributions and OC concentrations.

The chemical composition of the OM in the size fractions 20-53 µm and 53-250 µm was measured using a Bruker Avance III 200 spectrometer (Bruker, Billerica, USA) at a resonance frequency of 50.3 MHz using the cross-polarization magic angle spinning technique at a speed of 6.8 kHz. Contact time was 1 ms and recycle delay time was 0.4 s. The spectra were processed with a line broadening of 100 Hz, phase adjusted and baseline corrected. The spectra were separated into the four integration areas: carboxyl-C at 220-160 ppm, aryl-C at 160-110 ppm, O/N-alkyl-C at 110-45 ppm, and alkyl-C at 45-(-10) ppm. We used the ratio between alkyl-C

and O/N-alkyl-C as an indicator for the degree of decomposition of the OM (Baldock et al., 1997).

3.6 Fractionation by wet sieving

The isolation of aggregates using wet sieving was done according to Krause et al. (2018). Briefly, the pre-wetted field-fresh aggregates were isolated on a sieve tower under constant shaking (Kösters et al., 2013). The wet-sieved fractions < 250 µm will be further referred to as free wet-sieved size fractions. The fraction > 250 µm was sonicated at 60 J mL^{-1} according to Amelung and Zech (1999) to break down macroaggregates into microaggregates, which will be further referred to as occluded wet-sieved size fractions. To avoid re-aggregation, the fractions were shock frozen in liquid N_2 and freeze-dried after wet sieving (Siebers et al., 2018).

3.7 Microaggregate stability (tensile strength)

The mechanical stability of individual dry-crushed and wet-sieved (free and occluded) microaggregates was determined using the same high-resolution loading frame for material testing that was used for crushing the macroaggregates of < 2 mm (Zwick Roell AllroundLine, Zwick Roell, Ulm, Germany). The method is similar to the one described by Skidmore and Powers (1982). The loading frame was equipped with a high-resolution load cell with a capacity of 100 N (Xforce HP 100 N). We randomly selected 50 individual microaggregates of the size fraction 53-250 µm from each treatment and measured their weight with a high-precision balance (precision: 0.01 mg). After weighing the samples, they were placed on the table of the loading frame and a custom-made piston with a width of 500 µm was attached to the load cell of the frame. The location of the sample was checked with a Canon Eos 2000D digital camera, equipped with a macro lens. Subsequently, the distance between piston and table was reduced from 300 µm to 25 µm at a constant speed of 250 µm min^{-1} to ensure that the microaggregate is completely crushed (Figure S 4). The force required to crush each microaggregate was recorded and the work (the area of the load-displacement curves) was finally normalized by the aggregate's mass (mJ mg^{-1}). Since the aim of this study was to measure the stability of microaggregates (not of single particle grains), we only used the randomly selected specimen when they could reliably be identified as an actual microaggregate under the stereo microscope. If a specimen was identified as a large primary particle, it was omitted.

3.8 Molecular microbial community analysis

DNA was extracted using 300 mg of soil from the different aggregate size fractions with the Macherey Nagel NucleoSpin® Soil Kit (Macherey Nagel, Düren, Germany) following the manufacturer's instructions with minor modifications, which are outlined as follows. The mechanical disruption of the bacterial cells was performed for 60 s at 1200 rpm using the Fastprep96 instrument (MP Biomedicals, Santa Ana, USA). In the final step, DNA was resuspended in 30 µL of PCR-grade water. PCR amplification of the 16S rRNA gene, purification of PCR products, sample pooling and sequencing of the PCR products was done on the Illumina Hi Seq platform (2 x 250 bp reads) as described by Maarastawi et al. (2018).

3.9 Statistical analyses

We used SigmaPlot 11 and SPSS 25 to compute normality and equal variance tests and to analyze the variances. When significant at $p < 0.05$ a Tukey post-hoc test was applied to compare the means. Statistical analysis of the bacterial community data was done using R (R Core Team, 2017) with the Vegan (Oksanen et al., 2018) and Phyloseq packages (McMurdie and Holmes, 2013) as described earlier (Maarastawi et al., 2018). Sequences that were not assigned to the domains *Bacteria* or *Archaea* were excluded from the operational taxonomic unit (OTU) table, which was built using a 97 % sequence identity cut-off value for the classification of OTUs.

Alpha-diversity was analyzed as species richness based on the Chao1 index using a rarefied OTU table based on 7,057 reads per sample. Significant differences between samples were tested in the complete dataset using the non-parametric Kruskal-Wallis test with Nemenyi post-hoc tests (as a Shapiro-Wilk test revealed non-normal data distribution).

Beta-diversity is presented in non-metric multidimensional scaling (NMDS) plots based on a Bray-Curtis dissimilarity matrix, which was calculated from the OTU table. Significant differences between the groups of samples were evaluated by an analysis of similarity (ANOSIM) with 999 permutations, also based on the Bray-Curtis distance matrix. ANOSIM provides R-values in the range of 0 to 1, whereby 1 indicates clear differences in microbial community composition between predefined groups of samples, while 0 indicates the absence of differences. The reliability of the R-values is assessed based on p-values.

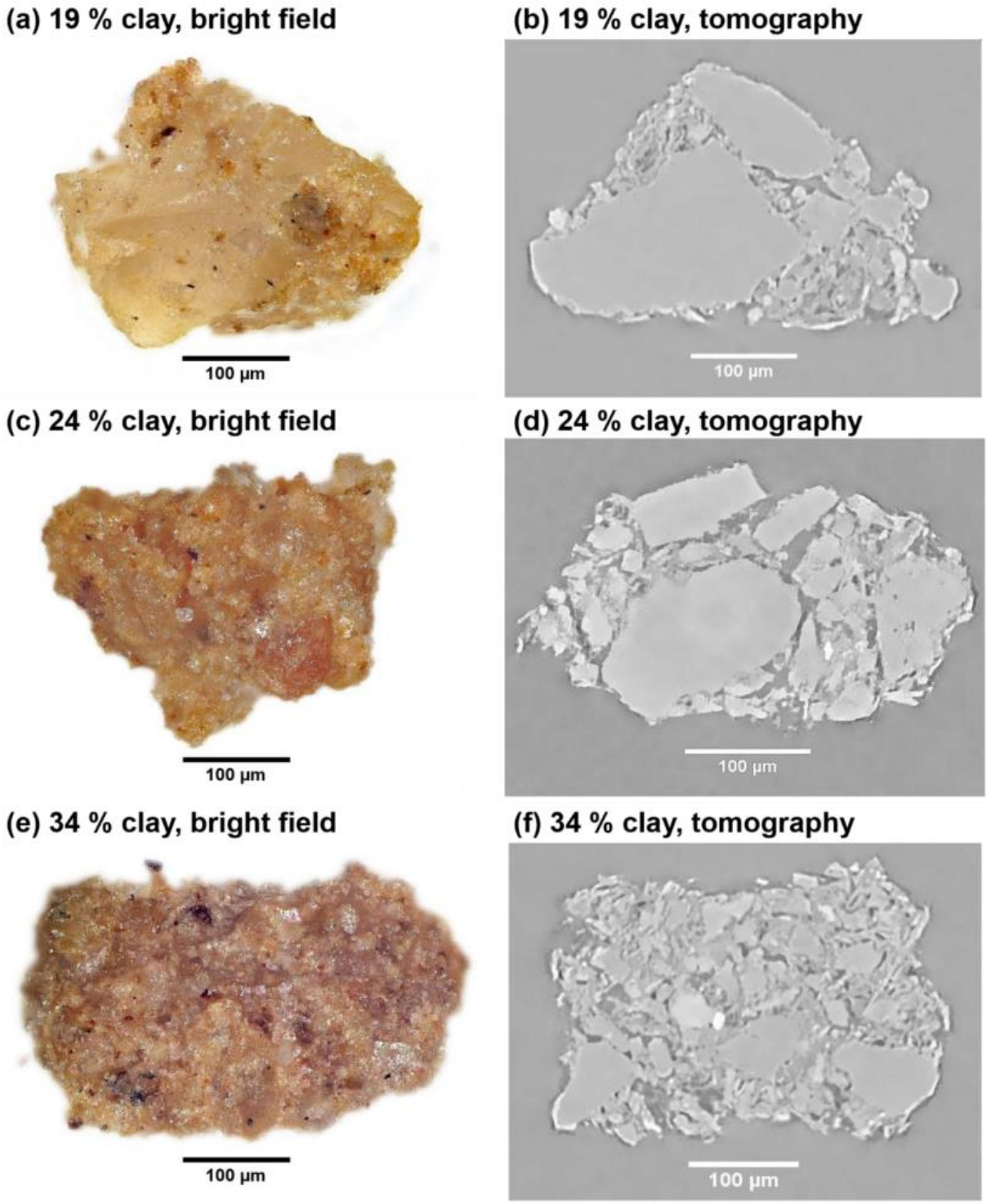
4. Results

4.1 Microaggregate structure as observed with microscopy and tomography

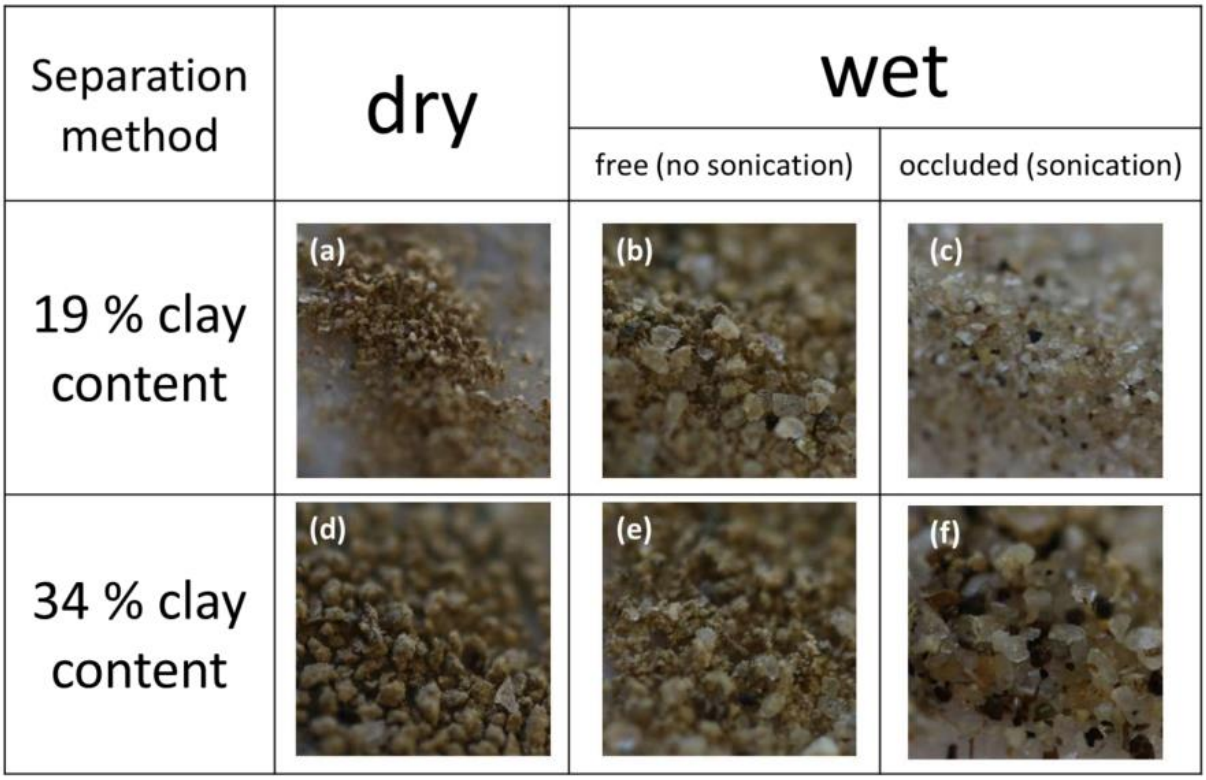
In the dry-crushed microaggregates from the soils with 19 % and 24 % clay, we found large sand grains contained within the aggregate structures in the large size fraction (Suppl. Fig. VII-2a-d). Exploratory CT scans of the dry-crushed samples confirmed this observation, namely that the content of large sand grains decreased with increasing clay content. These sand grains had diameters between 80 and 200 µm (Suppl. Fig. VII-2b, d, f). This finding was confirmed for 20 additional aggregates for each clay content, of which CT scans were also made (data not shown here). The aggregates in the 34 % clay soil mainly contained silt-sized primary particles although some sand-sized particles were also found (Suppl. Fig. VII-2e, f). The amount of free primary particles that were not integrated in a microaggregate was minor in all size fractions of the dry-crushed samples. In the samples isolated by wet sieving (with and without sonication), more free primary particles were found (Suppl. Fig. VII-3). Their number increased in the order dry crushing < wet sieving < wet sieving plus sonication (occluded) (Suppl. Fig. VII-3).

4.2 Particle and aggregate size distributions

The soil with 34 % clay content contained more macroaggregates > 250 µm than the soils with lower clay contents (Figure S 2). The microaggregate size fraction of < 53 µm was much lower for the soil with 19 % clay content compared to the soils with higher clay contents (Figure S 2). The high mass proportion of macroaggregates > 250 µm after the crushing procedure is likely explained by the uniaxial crushing. During uniaxial crushing, only one dimension of the aggregates can be controlled (here: height/the Y-axis), but in the X and Z axes, the samples can still be larger than 250 µm. The size distributions of the combined dry-crushed fractions (20-53 µm and 53-250 µm) showed a bimodal pattern with peaks at approximately 50 µm and 150 µm (Suppl. Fig. VII-4a). The mean object diameter of dry-crushed size fractions decreased significantly with increasing clay content from 163 µm to 125 µm (Table 1).



Suppl. Fig. VII-2: Incident light microscopy images and (b, d, f) tomograms of the dry-crushed microaggregate size fraction 53-250 µm from soils with different clay contents. With increasing clay contents, fewer (sand-sized) primary particles were observed. Resolution of the tomograms is 570 nm (b), 482 nm (d) and 656 nm (f).



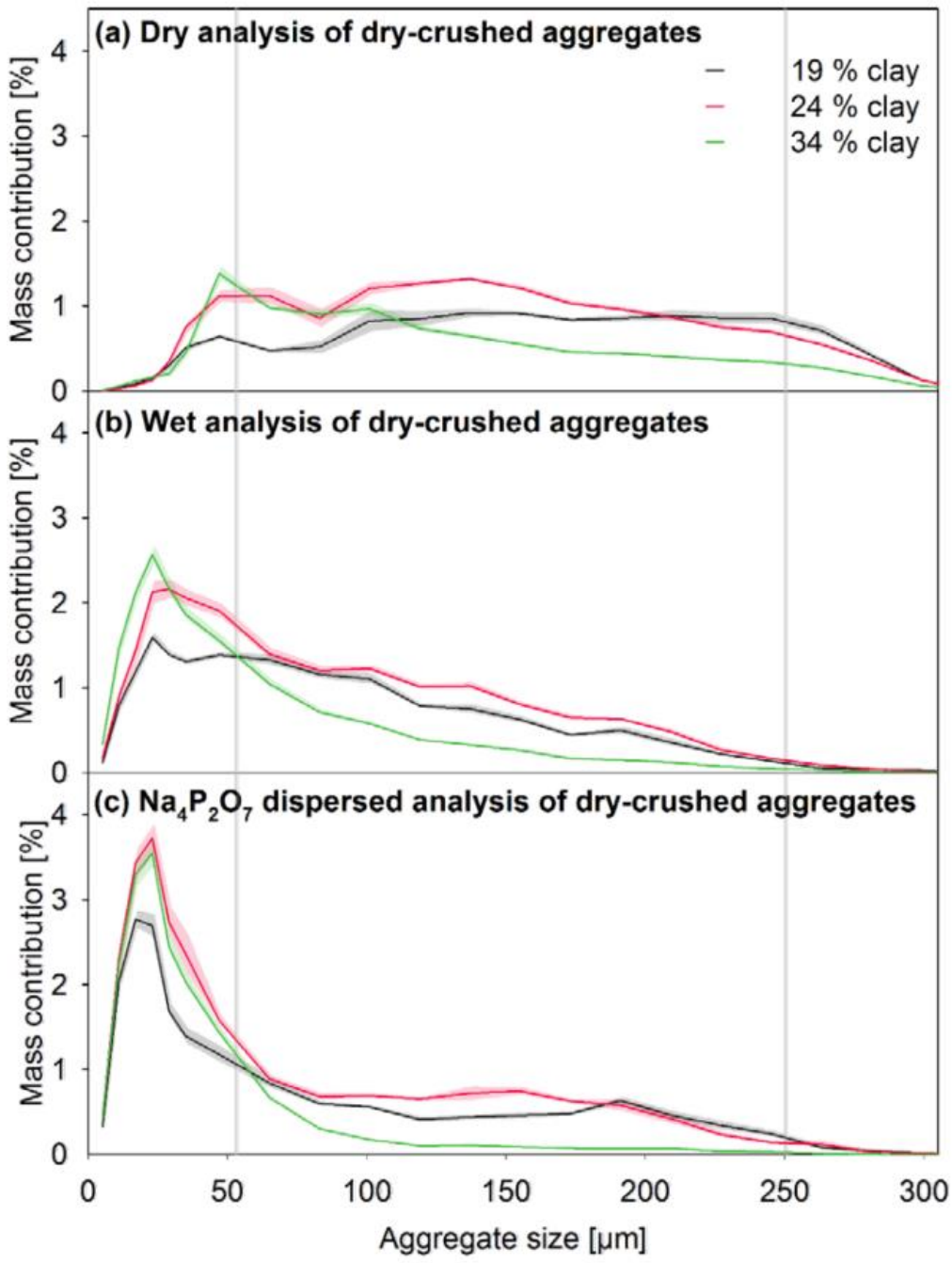
Suppl. Fig. VII-3: Macroscopic images of large microaggregates (53-250 µm) with a low (19 %, a-c) and high (34 %, d-e) clay content. Dry-crushed microaggregates (a, d) clearly exhibit a higher degree of aggregation of smaller particles, whereas microaggregates that were isolated by wet sieving without sonication (b, e) or with sonication (c, f) exhibited more free primary particles.

Tab. VII-1: Mean object sizes for the combined size fractions 20-53 µm and 53-250 µm (in µm; mean ± standard error; n = 5). Mean values of different clay contents that have the same lower case letter were not significantly different at $p < 0.05$. All analyses of variance were significant at $p < 0.001$.

Treatment	19 % clay		24 % clay		34 % clay	
dry-crushed, dry analysis	163.4 ± 3.2	a	145.4 ± 2.1	b	124.5 ± 1.8	c
dry-crushed, wet analysis	96.2 ± 0.8	a	96.3 ± 1.4	a	65.7 ± 1.1	b
dry-crushed, wet and $Na_4P_2O_7$-dispersed analysis	90.9 ± 1.7	a	83.5 ± 0.5	b	45 ± 0.7	c

When immersing these dry-crushed aggregates in water, the mean aggregate diameters decreased by approximately 60 µm (Table 1). The size distribution shifted to a more unimodal pattern with a peak at approximately 25 µm (Suppl. Fig. VII-4b). The soil with 34 % clay showed the smallest objects by comparing the dry to the wet measurement, which indicated most aggregate breakdown by water immersion (Suppl. Fig. VII-4b, Table 1). When dispersing the aggregates further, we found almost no sand-sized primary particles in the soil with highest

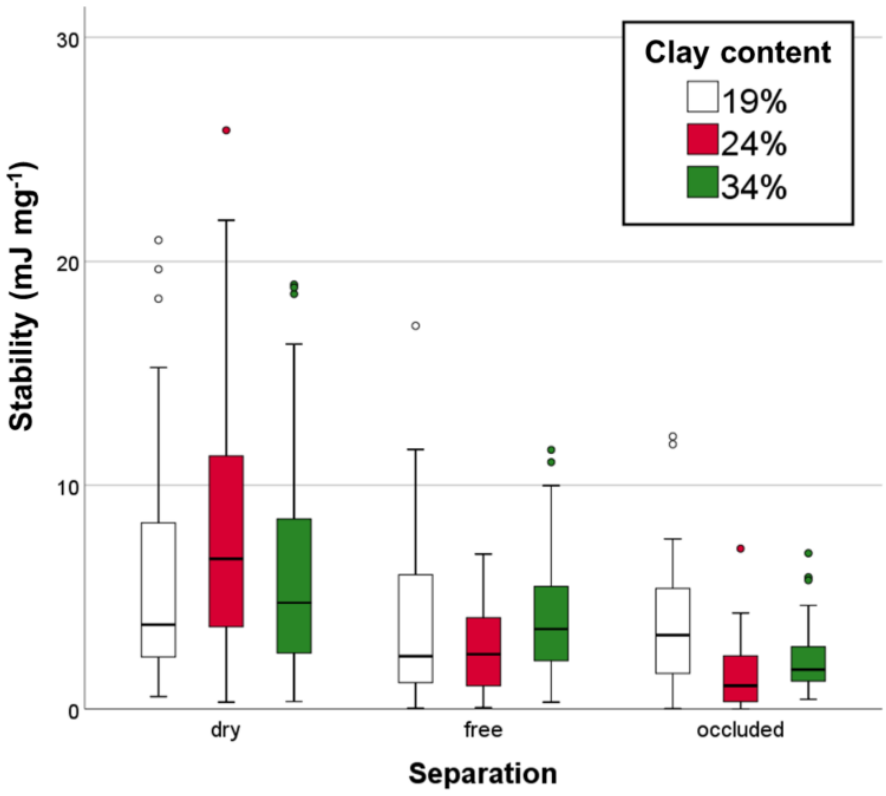
clay content (34 %), while the soil with 19 % clay also showed less silt-sized particles (Suppl. Fig. VII-4c).



Suppl. Fig. VII-4: Mass contributions of dry-crushed microaggregates to the bulk soil analyzed (a) in a dry state, (b) in a wet state, and (c) in a wet and dispersed state. The size distribution was merged from the two individual size distributions of the size fractions 20-53 µm and 53-250 µm according to their relative mass contribution to the bulk soil (mean ± standard error).

4.3 Mechanical soil aggregate stability (tensile strength)

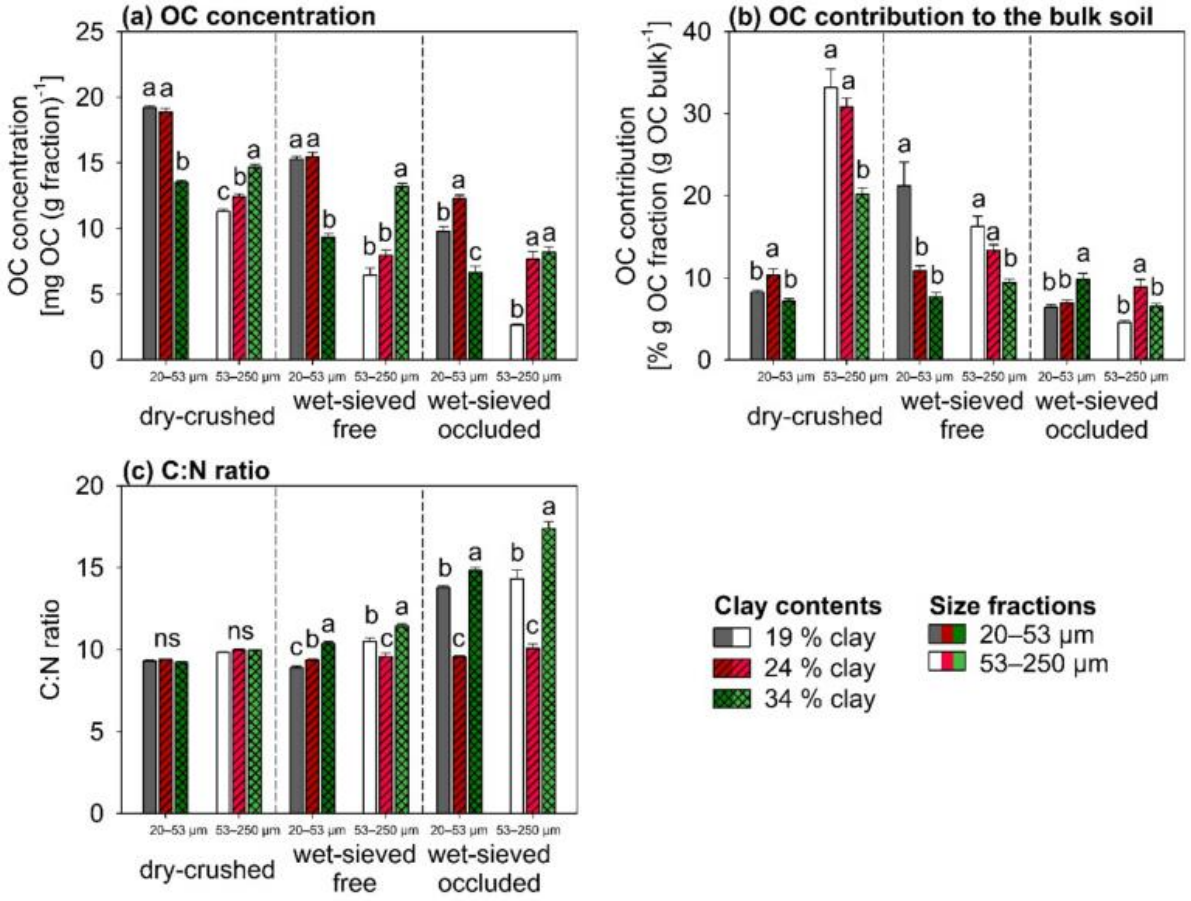
The mechanical stability of the microaggregates was in a similar range across all clay contents within one isolation method. No significant differences were observed for microaggregates obtained by different isolation methods or between soils of different clay contents using the same method. Despite the lack of statistically significant differences, the mechanical stability showed a clear tendency to decrease from dry-crushed to wet-sieved (free) to occluded microaggregates (Suppl. Fig. VII-5). This indicates that the structure of the samples becomes more homogeneous after increased aggregate breakdown, which is shown by the variability of tensile strengths that decreases in the same direction. It should be noted that although we measured approximately 50 replicates per isolation method and clay content, these samples were hand-picked under a stereo microscope. For a truly representative result, it would be necessary to measure the stability of all microaggregates that are isolated from a given number of macroaggregates and that is much higher than 50. However, the high number of replicates makes us confident that the high variability of our results actually represents the real degree of structural heterogeneity of the microaggregates that we investigated.



Suppl. Fig. VII-5: Mechanical stability of microaggregates with different clay contents obtained with different isolation methods (n = 43 to 50). Circles represent outliers. Extreme outliers (> 3 SD) were omitted.

4.4 Organic matter content and chemical composition of microaggregates

The OC concentration in the medium-sized fractions (20-53 µm) was generally lowest in the soils with 34 % clay compared to higher OC concentrations in the soils with 19 % clay for all isolation methods. In contrast, the OC in the large fractions (53-250 µm) increased with increasing clay content for all isolation methods (Suppl. Fig. VII-6a). When comparing the OC concentrations between aggregates obtained based on the different isolation methods, they decreased in the order dry-crushed > wet-sieved free > wet-sieved occluded (Suppl. Fig. VII-6a).



Suppl. Fig. VII-6: (a) OC concentration, (b) OC contributions and (c) C:N ratios comparing dry-crushed size fractions 20-53 µm and 53-250 µm with wet-sieved free and wet-sieved occluded size fractions (mean ± standard error; n = 5). Means with the same letter in the same isolation method and size fractions were not significantly different. Data of the wet-sieved free and wet-sieved occluded fractions adapted from Krause et al. (2018).

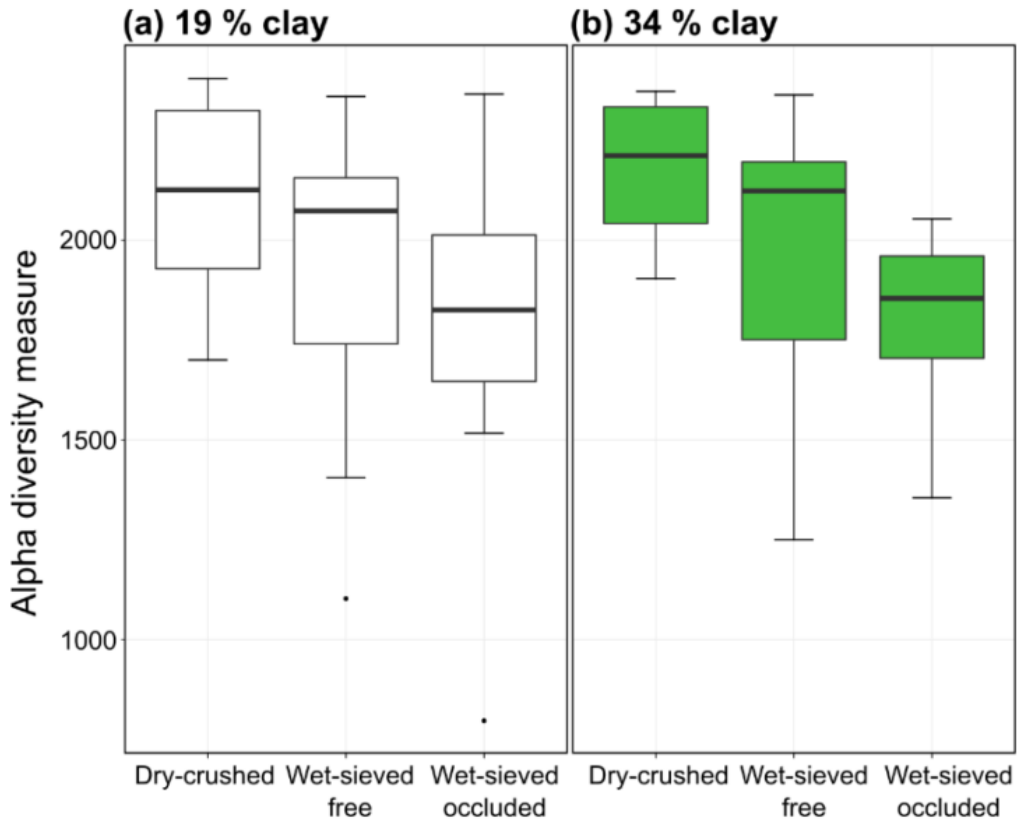
In the dry-crushed fractions, the OC contribution to the bulk OC was higher in the large size fraction of the 19 % and 24 % clay soil compared to the 34 % clay soil (Suppl. Fig. VII-6b). In the wet-sieved free fractions, the OC contributions of the medium and large size fractions were higher with lower clay contents (Suppl. Fig. VII-6b). In the wet-sieved occluded fractions,

however, the OC contributions of the medium size fraction was higher in the 34 % clay soil compared with the 19 % and 24 % clay soil (Suppl. Fig. VII-6b). The mass contributions of the dry-crushed and wet-separated fractions (free aggregates) were in a comparable range but deviated for the wet-separated and sonicated fractions (occluded aggregates, Figure S 5). The OC concentrations of the size fractions were similar when the OC concentrations of the medium and large size fractions were calculated, while excluding the medium to coarse silt and sand grains according to their mass contributions (Table S 2).

The C:N ratios were higher for the large size fraction compared with the medium size fraction (Suppl. Fig. VII-6c). This difference was, however, smallest between the dry-crushed fractions, which did not show any differences across the clay content. The wet-sieved fractions showed lower C:N ratios in the 19 % clay content compared with 34 % clay. When analyzing the OM composition by ^{13}C NMR, we observed more O/N-alkyl C in the dry-crushed size fraction 53-250 µm than in the size fraction 20-53 µm (Table S 4).

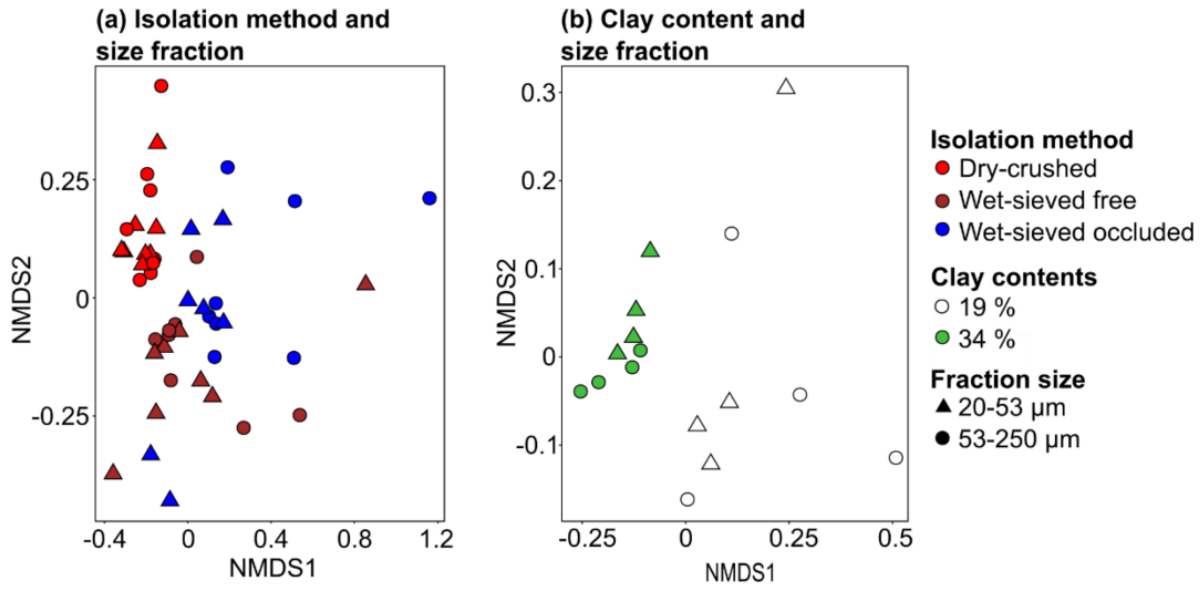
4.5 Composition and diversity of bacterial communities in the soil microaggregates

We compared the bacterial community composition of the large and medium size fractions that were obtained from the soils of the lowest (19 %) and highest (34 %) clay content with the different isolation methods. Here, we focused on the highest and lowest clay content because the generation of sufficient dry-crushed material for the molecular work was rather laborious and because sample material was limited. Analysis of the alpha diversity based on the Chao1 index revealed that the bacterial diversity was lower in the wet-sieved occluded fraction compared to the dry-crushed fraction ($p < 0.01$), while no significant difference was seen between the dry-crushed fraction and wet-separated free fraction (Suppl. Fig. VII-7).



Suppl. Fig. VII-7: Comparison of the alpha diversity based on the Chao1 diversity index between dry-crushed and wet-separated (wet-sieved free and wet-sieved occluded) fractions (including the size fractions 20-53 µm and 53-250 µm) for two soils with (a) 19 % clay and (b) 34 % clay.

Neither clay content nor size of the fraction affected alpha diversity significantly. Differences in bacterial community composition between size fractions (i.e. beta diversity) were assessed in NMDS plots. This revealed that the isolation method had a significant effect on the bacterial community composition, evident from the clustering of size fractions in the plot according to the isolation methods (Suppl. Fig. VII-8a). The dependence on the isolation method was statistically confirmed by an analysis of similarity (ANOSIM), resulting in an R-value of 0.51 ($p < 0.001$). Based on this finding, the effects of clay content and fraction size were further compared between dry-crushed and wet-fractionated samples. This comparison revealed a much stronger effect of clay content on bacterial community composition in the dry-crushed fractions (ANOSIM $R = 0.767$, $p = 0.001$; Suppl. Fig. VII-8b) compared to the wet-separated fractions, where a weaker effect was seen in the wet-sieved free fraction and no effect in the wet-sieved occluded fraction (ANOSIM $R = 0.157$, $p = 0.005$). An effect of the fraction size on the bacterial community composition was again not detectable, regardless of the isolation method (Suppl. Fig. VII-8a, Table S 3).



Suppl. Fig. VII-8: NMDS plots comparing (a) isolation method and size fraction and (b) clay content and fraction size of dry-crushed fractions. NMDS plots were calculated based on Bray-Curtis distance matrices, derived from 16S rRNA gene amplicon data.

5 Discussion

5.1 Comparing dry crushing and wet sieving on the basis of mechanical stability, organic carbon concentrations and bacterial diversity

Depending on the isolation method, the tensile strength and variability of soil fractions in the size range of microaggregates decreased in the order dry-crushed > wet-sieved free > wet-sieved occluded (Suppl. Fig. VII-5). When comparing the isolation methods, the OC concentrations and OC contributions of the size fractions exhibited a similar tendency and decreased in the same order (Suppl. Fig. VII-6). The mass contributions of dry-crushed and wet-sieved free fractions were in a similar range (Figure S 5). The differences in the OC content are related to different aggregate failure mechanisms addressed by dry crushing in comparison to wet sieving resulting in different isolated subunits. While dry crushing led to the isolation of size fractions where primary particles were still observed within aggregate structures (Suppl. Fig. VII-2), wet sieving produced size fractions containing free primary particles outside of aggregates (Suppl. Fig. VII-3). For the mechanical stability measurements, individual aggregates were manually selected, whereas free primary particles were not included. The higher content of sand-sized particles in the dry-crushed fractions led to a higher variability of the tensile strength (while they were absent in the wet-sieved samples, sand grains still contributed to

the overall aggregate stability in the dry-crushed samples). Hence, with respect to the effect of isolation method on aggregate stability, our hypothesis could not be confirmed.

The isolation of defined size fractions from larger aggregates necessitates disaggregation and breakdown forces for the separation into smaller units. Mechanical separation by uniaxial dry crushing or dry sieving appears to generate different aggregate subunits than wet sieving, due to the different failure mechanisms (mechanical vs. hydraulic stresses). This has a great influence on aggregate properties. The size composition of wet-sieved size fractions was shown to be highly reproducible by Graf-Rosenfellner et al. (2018). This homogeneity, however, appears to be the product of the wet sieving procedure with sonication.

Dry separation avoids a change in physical, chemical and biological characteristics that can be observed during drying and wetting. These can be (as reviewed by Kaiser et al. (2015)) the formation of new interactions between minerals and OM, the killing of microorganisms and shifts in their community structure and an increase of mineral surface acidity and hydrophobicity (possibly affecting aggregate stability, cf. Ibrahimi et al. (2019)). The degree to which these characteristics are altered by wetting and drying depends on various factors, like soil depth (Kaiser et al., 2015), land use (Fierer and Schimel, 2003), texture and OM content (Albalasmeh and Ghezzehei, 2014), sodium and calcium cation configuration (Aquino et al., 2011), pH value (Kang and Xing, 2008) and drying speed (Kemper and Rosenau, 1986). All of these influences alone are difficult to quantify, which is why (repeated and/or unnecessary) wetting and drying should be avoided to preserve the natural properties of soil aggregates and to prevent the creation of artifacts.

The comparison of isolation methods demonstrated that while wet-sieved fractions were found to contain more free primary particles and fine-sized aggregates, dry-crushed fractions contained more aggregates with inclusions of primary particles. This leads to more heterogeneous aggregate architectures within the size fractions isolated by dry crushing. This heterogeneous architecture is reflected by a higher bacterial alpha diversity in the dry-crushed fractions (Suppl. Fig. VII-7). Significant differences in dependence on the isolation method were also observed in the bacterial community composition (Suppl. Fig. VII-8a). It is known that the use of classical methods for wet sieving and dry sieving (not comprising dry crushing) each come with specific influences on the bacterial community composition (Wilpiszeski et al., 2019). These differences are likely related to habitat heterogeneities of the respective aggregate fractions. In addition, differences in the bacterial community composition may result

from the different methodological procedures, i.e. the immersion in water during wet sieving, which may cause the dislodgement of bacterial cells from microaggregates. The loss of bacterial cells by wet sieving steps is likely affecting different species to a different extent, considering that the interactions between bacterial cells and soil constituents such as mineral particles are known to vary between species or even strains (Ams et al., 2004; Hong et al., 2012; Huang et al., 2015; Krause et al., 2019). Specific strains may be more easily lost during the wet sieving process compared to others, which may have contributed to a reduced diversity on wet-sieved microaggregates, especially for the wet-sieved occluded microaggregates, which are exposed to wet sieving twice and additionally treated by ultrasound. In addition, the wet-sieving protocol includes a lyophilization step, but we consider this as minor source for bias, as we observed no significant effects on alpha or beta diversity when testing the effect of lyophilization on bacterial community composition with a subset of bulk soil samples independently (see Fig. S 7). Undoubtedly, the air-drying step of the soil prior to the dry crushing procedure also has an influence on the bacterial community, but this can be regarded as less intrusive for DNA-based studies and is causing less bias compared to the full wet-sieving protocol. Dry crushing might therefore be a useful alternative when microaggregates need to be isolated while better maintaining bacterial community composition. It should be noted that the methodological impact of the different fractionation procedures may have different effects on microbial respiration or enzyme activities, which remain to be evaluated in detail. Likewise, future studies should be performed to assess systematically the identity of the taxa that are primarily affected by different fractionation methods.

Based on our findings, i.e. the isolation of different structures during dry crushing and a potential loss of cells due to the wet sieving procedure, our initial hypothesis of a higher bacterial diversity in the dry-crushed samples was confirmed. This is in accordance with studies that compared the impact of wet sieving with dry sieving on microbial parameters (Blaud et al., 2017; Nahidan and Nourbakhsh, 2018; Trivedi et al., 2017). Likewise as in this study, Blaud et al. (2017) found major differences in the microbial community composition between wet-sieved and dry-sieved fractions, whereas the effect of the size of a fraction was minor. Besides the reduction of sources introducing methodological bias, aggregate properties, i. e. the differences in failure mechanisms and structures observed in this study might explain some of the previously observed differences in the microbial community between dry separation and wet sieving techniques (Blaud et al. 2017; Bach and Hofmockel 2014). The recommendation

of earlier studies to employ wet sieving when focusing on long-term changes in OM and enzyme activity is, therefore, related to a preferential isolation of fine particle and OM-rich aggregates (Nahidan and Nourbakhsh, 2018). Our results show that the dispersion and loss of sand-sized particles by wet sieving from intact aggregate structures may distort the transfer of findings from the isolated microhabitats and their associated microbial communities to the intact bulk soil.

5.2 How clay content affects the soil microstructure of dry-crushed microaggregates

Since differences in texture may lead to different aggregate architectures, we analyzed the microaggregates isolated by dry crushing from soils with different clay contents. A bimodal size distribution of dry-crushed microaggregates reveals the existence of preferential microaggregate sizes with diameters of approximately 50 µm, which were more abundant in the 34 % clay soil, and approximately 150 µm, which were more abundant in the 19 % clay soil (Suppl. Fig. VII-4a). The smaller aggregates in the soil with 34 % clay compared to those in the soil with 19 % clay can be explained by a smaller size of the primary particles in soils with high clay content. The mechanical breakdown of the 34 % clay soil into smaller structural units indicates a multitude of failure planes compared to the 19 % clay soil, which broke down into larger structures as a result of having fewer failure planes.

Increasing aggregate diameters in dry-crushed fractions of low-clay soils are in contrast to the aggregate relationships found by wet sieving. In a previous study using wet sieving on the same gradient, most water-stable microaggregates were found to be of approximately 30 µm diameter independent of differences in clay content (Schweizer et al., 2019). In the high-clay soils, more water-stable macroaggregates > 250 µm were found, whereas microaggregates were larger in the size range of 50-180 µm (Schweizer et al., 2019). Accordingly, various earlier studies that applied wet sieving found larger water-stable aggregates in soils with higher proportions of 2:1 clay minerals (Amézketa, 1999; Bronick and Lal, 2005; Six et al., 2000b). During wet sieving, soil structures are mostly dispersed through slaking and the swelling of phyllosilicates. By contrast, the dry crushing method breaks soil structures along mechanical planes of weakness (Kristiansen et al., 2006).

The size distribution measurements of dry-crushed fractions after water immersion and $Na_4P_2O_7$-dispersion show which sizes of water-stable units and primary particles are contained in dry-separated aggregates (Suppl. Fig. VII-4b, c). The dry-crushed microaggregates in the lower clay soils (19 and 24 % clay) consisted of more sand-sized particles with sizes around

100-250 µm in contrast to the 34 % clay soil. The comparison of the particle size distributions in dry, wet, and dispersed state indicate that a majority of these sand-sized particles formed parts of dry-crushed soil aggregates. This was also visible in both, microscopy images and tomography scans (Suppl. Fig. VII-2), suggesting that the inclusion of sand-sized particles increased the microaggregate diameter in the soils with lower clay contents. In an earlier study using wet-sieved fractions, free primary particles > 100 µm were observed not to form water-stable microaggregates (Schweizer et al., 2019). In this study, we found such sand-sized primary particles integrated in aggregates of dry-crushed size fractions. Accordingly, primary particles with diameters > 100 µm are stable aggregate compounds when samples are subjected to dry crushing but may be detached from fine particles during wet sieving procedures.

The effect of isolation method on the particle size compositions is a consequence of the different failure mechanisms of wet sieving (hydraulic) and dry crushing (mechanical). It seems that wet sieving addresses more (and probably other) points of weakness, resulting in different microaggregate subunits. Fine-sized aggregates preferentially remain stable during the isolation of fractions with wet sieving. Such fine aggregates are reflected by the unimodal size distribution at approximately 25 µm after immersing the dry-crushed microaggregates in water (Suppl. Fig. VII-4b), which is similar to the preferential microaggregate size of wet-sieved microaggregates found in a previous study of the same soils (Schweizer et al., 2019). When upscaling findings based on wet-sieved aggregates, it is therefore important to consider that these do not fully represent the soil structure that was present under field conditions. Therefore, if natural aggregate structures are of interest for the isolation of fractions that include their original sand-sized primary particles, dry crushing shows advantages over wet sieving.

5.3 Mechanical stability of microaggregates across a texture gradient

The mechanical stability of individual microaggregates from all clay contents showed a similar range and a high variability (Suppl. Fig. VII-5). The initial hypothesis about the influence of clay content on aggregate stability could therefore not be confirmed. Since microaggregates from the soil with a lower clay content contained more sand-sized primary particles, a similar range of stability suggests that sand-sized particles exerted the same influence on mechanical stability at all clay contents. Alternatively, sand-sized particles within microaggregates might also have less influence on their stability, since aggregates broke along their weakest mechanical failure planes that might be located in parts of the aggregate with fine particles. The influence of the particle arrangement in a soil aggregate on its mechanical stability warrants further

studies to determine the failure planes related with the aggregate breakdown upon crushing. For these future studies, it is sensible to also use air-dry samples because it is a realistic assumption for near-surface soils and easy to standardize, providing samples with similar matric potentials, which is one of the major influences on soil stability (Horn and Peth, 2012). Our results show, for the first time, that it is possible to determine the stability of an individual microaggregate specimen in a loading frame and give a first overview of the range of mechanical stability and the relationship of microaggregates with soil clay content and the isolation method.

5.4 Organic matter allocation in microaggregates

The OC concentrations in large microaggregates (53-250 µm) were found to increase in the soils with higher clay content and were lower in the soil with 34 % clay compared to the soil with 19 % clay in medium-sized microaggregates (Suppl. Fig. VII-6a). This was connected to the inclusion of primary particles in size fractions of the low clay soils and confirmed our initial assumptions. When the sand- and silt-sized primary particles were excluded from the fraction masses included in the measured OC concentrations, both the large and medium size fractions had similar OC concentrations. This suggests that the contribution of fine particles in aggregates is strongly related with the OC contents. Fine mineral particles provide reactive mineral surfaces to stabilize OM or larger structures that occlude OM (Baldock and Skjemstad, 2000; Ransom et al., 1998). In turn, the proportion of larger primary particles occupying space within aggregates reduces the OC concentrations. This can also explain the difference of OC concentrations between the size fractions (Suppl. Fig. VII-6a): The primary particles contained in the medium size fraction might occupy less space than the larger primary particles in the large size fraction. The OM composition was different between the two dry-crushed size fractions but similar across the clay content (Suppl. Fig. VII-6c; Table S 4). A higher C:N ratio and a lower alkyl:O/N-alkyl ratio indicated OM to be in a less decomposed state in the large compared to the medium size fraction. This corroborates previous studies that used wet-sieved size fractions (Fernández-Ugalde et al., 2013; Schweizer et al., 2019), despite the relatively low signal-to-noise ratio of the NMR spectra in this study (Figure S 6, Table S 4). Larger fraction sizes might simply include more or larger pore spaces, which could be filled with OM, inhibiting its further decomposition. This assumption has to be confirmed by the analysis of the three-dimensional structure of the aggregate pore systems. The lower C:N ratio of the medium size fraction might also be related to higher amounts of mineral-associated ammonium, which was

shown to correlate positively with clay content (Jensen et al., 1989). The C:N ratios were, however, similar within the size fraction and did not increase in soils with higher clay contents, which contained more clay- and silt-sized primary particles. Therefore, the C:N ratios were mostly related to the decomposition of OM instead of mineral-associated ammonium. The difference in OM composition between the fraction sizes was not reflected by the microbial community composition, which was similar regardless of the microaggregate size. While the OC concentrations of the size fractions were mostly related with the proportion of fine primary particles, the OM composition differed between the size fractions depending on its capacity to stabilize OM.

It seems plausible that both, wet sieving and dry crushing, yield different results for the OC distribution of the different aggregate size fractions. During wet sieving (especially with sonication), large primary particles are dispersed from fine particles (containing the most OC) (Nahidan and Nourbakhsh, 2018). This leads to an underestimation of the OC content in the largest fraction and an overestimation of OC in the smallest fraction. On the other hand, if larger quartz grains are ground into smaller primary particles during dry crushing, their resulting increased abundance in the smaller fractions will probably dilute the smallest fraction, leading to an underestimation of the real OC content. More studies are needed in order to quantify the relevance of each of these two mechanisms for different OC concentrations and substrates, i.e. sand contents, possibly also comparing dry sieving with and without prior crushing.

5.5 Microbial community composition in dependence on soil clay content

In the dry-crushed fractions, we observed stronger differences in bacterial community composition in dependence on soil clay content (Suppl. Fig. VII-8b), while no detectable differences between microaggregate sizes were seen regardless of the applied fractionation method. This is likely related to our findings on a changing size composition of the microaggregates as indicated by an increasing proportion of sand-sized primary particles in microaggregates from soils with lower clay content. The fact that no differences were seen in bacterial community composition between fraction sizes 20-53 µm and 53-250 µm seems to contradict the findings of some other studies, where distinct bacterial communities in micro- and macroaggregates were seen (Davinic et al., 2012; Fox et al., 2018; Trivedi et al., 2017; Upton et al., 2019). This effect might therefore be scale dependent, meaning that while differences between micro- and macroaggregates can be found, they do not exist within the microaggregate size fractions that we investigated in this study.

5.6 Using dry crushing for the isolation of aggregates in future studies

The dry crushing procedure enables addressing different research questions that expand current aggregate separation methods, like the isolation of aggregates without the dissolution of water extractable OM or the quantification of the mechanical stability of individual aggregates. To adopt the dry crushing procedure, a loading frame is not a necessity, since one does not necessarily need to record the applied force. Any device that can be used to crush soil macroaggregates by reducing the distance of two planar surfaces to a defined threshold (250 µm in this case) would suffice to isolate fractions. For example, a modified bench vise could be used. Alternatively, a metal block with a 250 µm deep cavity could be manufactured. However, this would not allow recording of load-displacement curves, which indicates individual tensile strengths of aggregates and can reveal the crushing of individual primary particles (see 4.3). The utilization of different sized spacers between planar surfaces would allow researchers to isolate any aggregate size that is of interest for a particular study.

6 Conclusions

We showed that dry crushing allows the isolation of size fractions that are characterized by a more variable mechanical stability compared to isolation by wet sieving. This is related to a higher content of sand-sized particles in dry-crushed aggregates according to different aggregate failure mechanisms during dry uniaxial mechanical crushing compared to wet sieving. We found that dry-crushed aggregates contained sand-sized primary particles, whereas the wet-sieved fractions contained more free primary particles released from aggregates. These differences in aggregate properties were accompanied with differences in bacterial colonization. Besides, the additional re-wetting and sonication during wet sieving probably removed bacterial cells, leading to a lower bacterial diversity in the occluded microaggregate fraction compared to the microaggregates obtained upon dry crushing. We suggest dry crushing as an alternative isolation procedure that is better preserving the bacterial associations with minerals. Clearly, the choice of the isolation method is reflected in microbial community composition, which should be considered in future studies and when comparing data between studies.

On average, the inclusion of sand-sized particles in dry-crushed aggregates led to 40 µm larger microaggregate structures when comparing the soils with low and high clay content. The dispersion during wet sieving equalized the size distribution with most objects at a diameter of approximately 25 µm. The OC concentrations in the size fraction 53-250 µm increased with the clay content of soils. This is explained with a decreasing proportion of sand-sized primary particles in the aggregates from the soils with higher clay contents. The wet-sieved size fractions reflected similar patterns of OC concentration changes across clay content and fraction size. The sand-sized and silt-sized particles within aggregates are dispersed during wet sieving. Therefore, aggregates in wet-sieved size fractions contain more clay-sized and silt-sized particles. If the aim of aggregate isolation is to isolate intact structures including sand-sized primary particles, dry crushing can be recommended.

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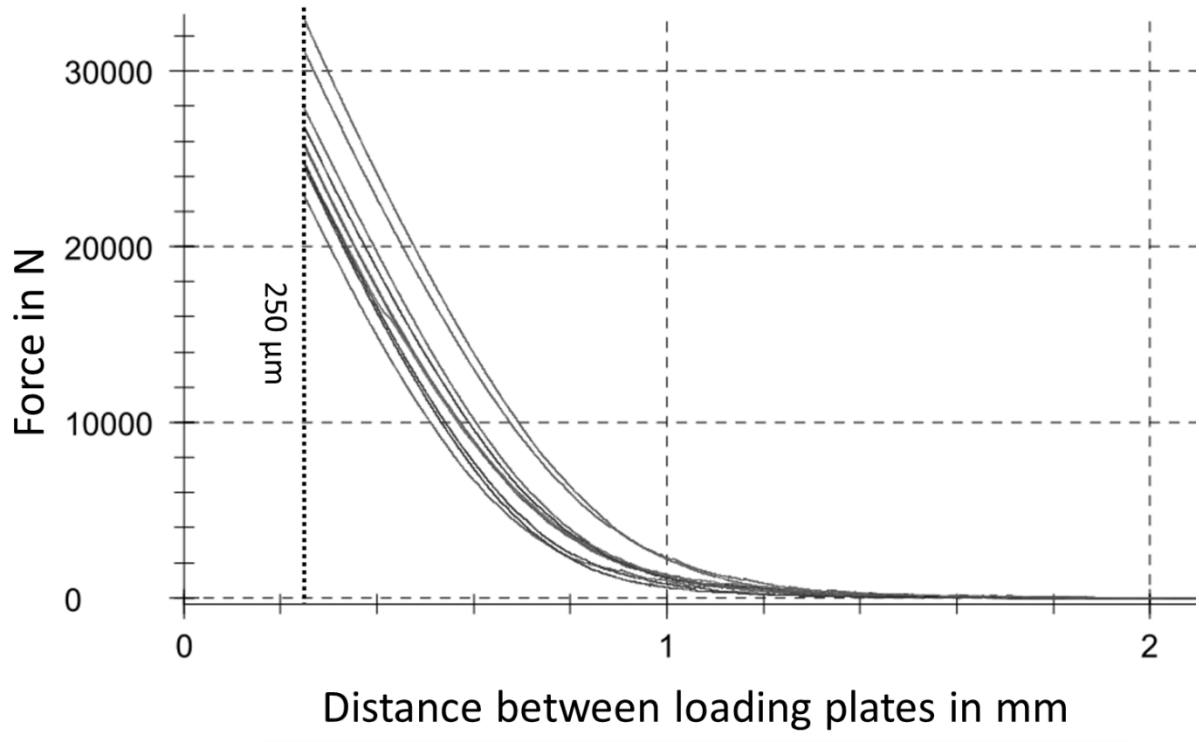
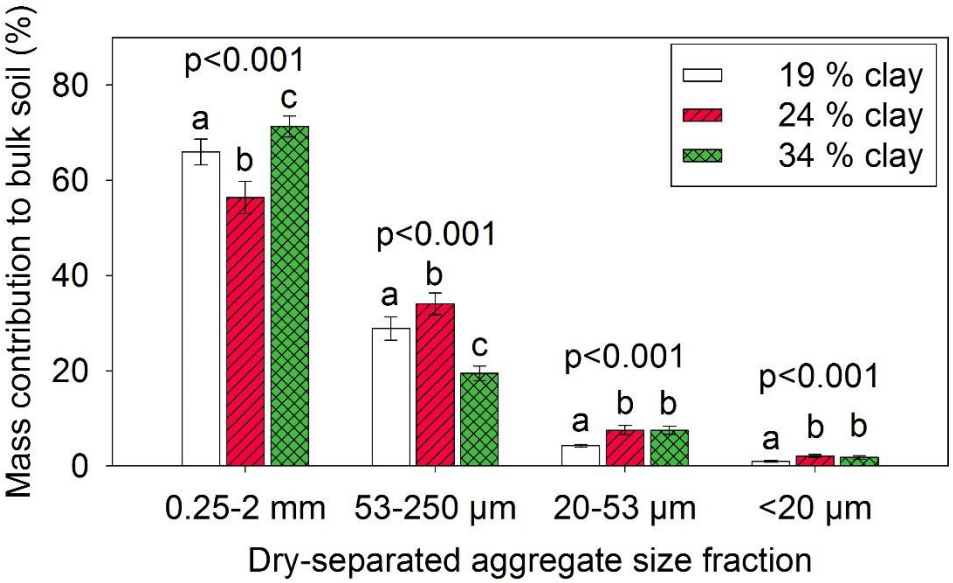
Supplementary materials

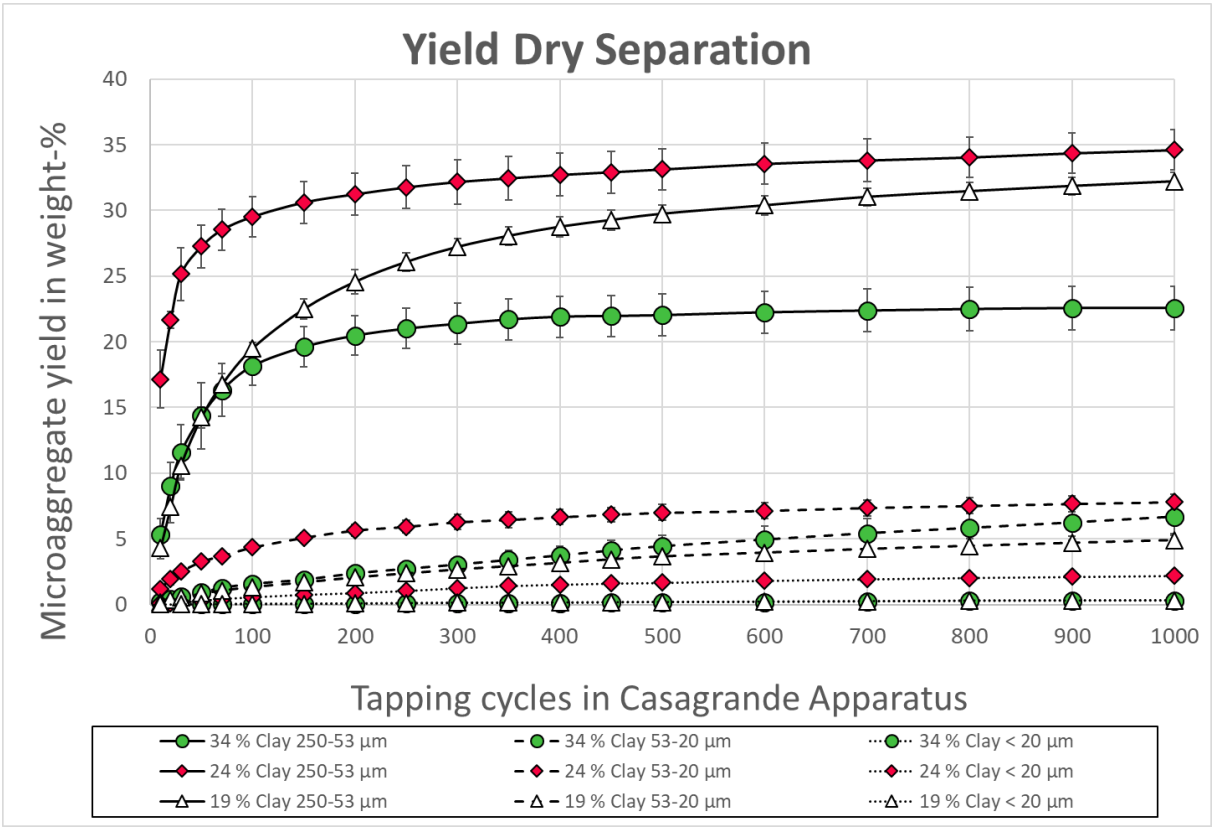
Supplementary figures



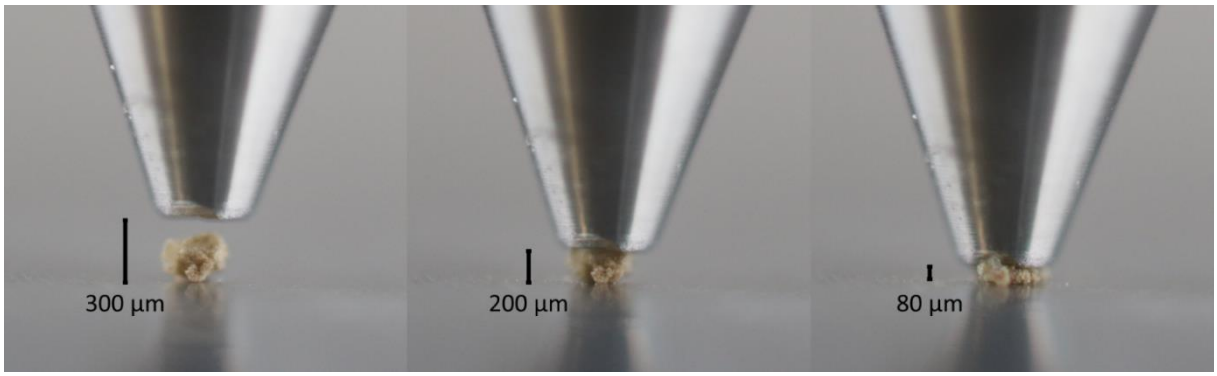
Supplementary Figure 1: Load-displacement curve of the 19 % clay sample. The lack of steep peaks indicates that the breaking of sand-sized primary particles did not occur during dry crushing.



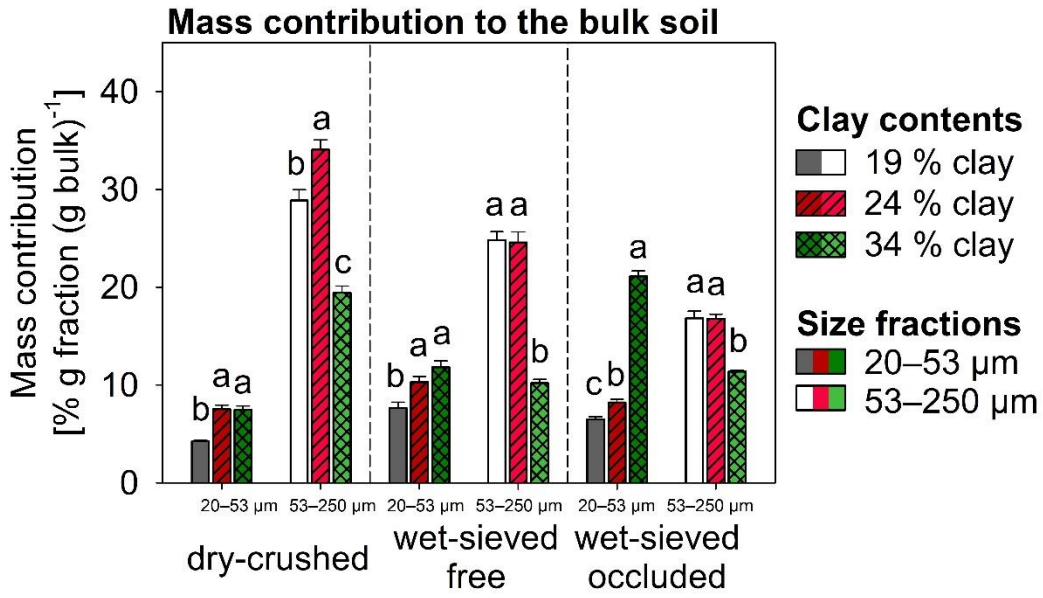
Supplementary Figure 2: Mass contributions of the dry-crushed aggregate size fractions to the bulk soil of three different soil textures (n = 5).



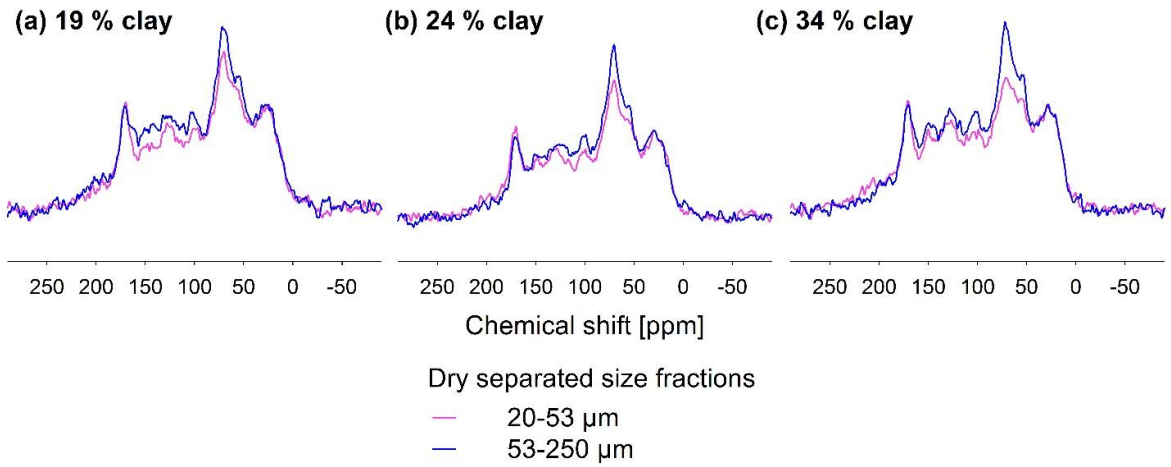
Supplementary Figure 3: Yield of the three microaggregate fractions after 1000 tap cycles in the Casagrande Apparatus for all three clay contents.



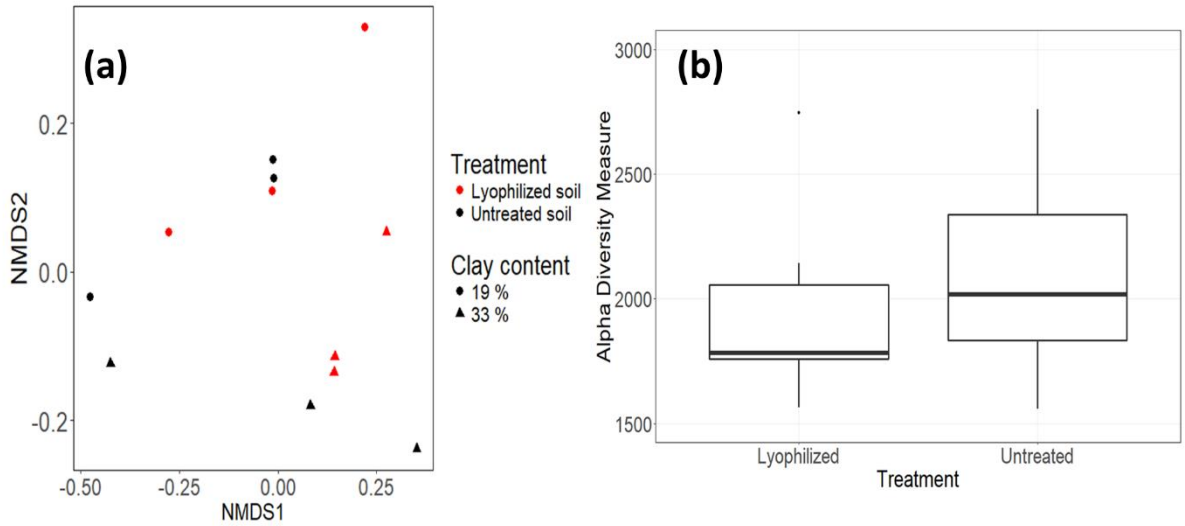
Supplementary Figure 4: Mechanical stability measurement through load-displacement curves of an individual microaggregate of the size fraction 53-250 µm (19 % clay content) in a Zwick Roell loading frame. Scale bar shows distance between piston and table in µm. An animated GIF of this measurement can be found at https://doi.org/10.6084/m9.figshare.12055722.v1.



Supplementary Figure 5: Mass contribution comparing dry-crushed with wet-sieved free and wet-sieved occluded size fractions of 20-53 µm and 53-250 µm. The samples were summarized to three main clay contents (mean ± standard error; n=5). Data of the wet-sieved free and wet-sieved occluded fractions adapted from Krause et al. (2018).



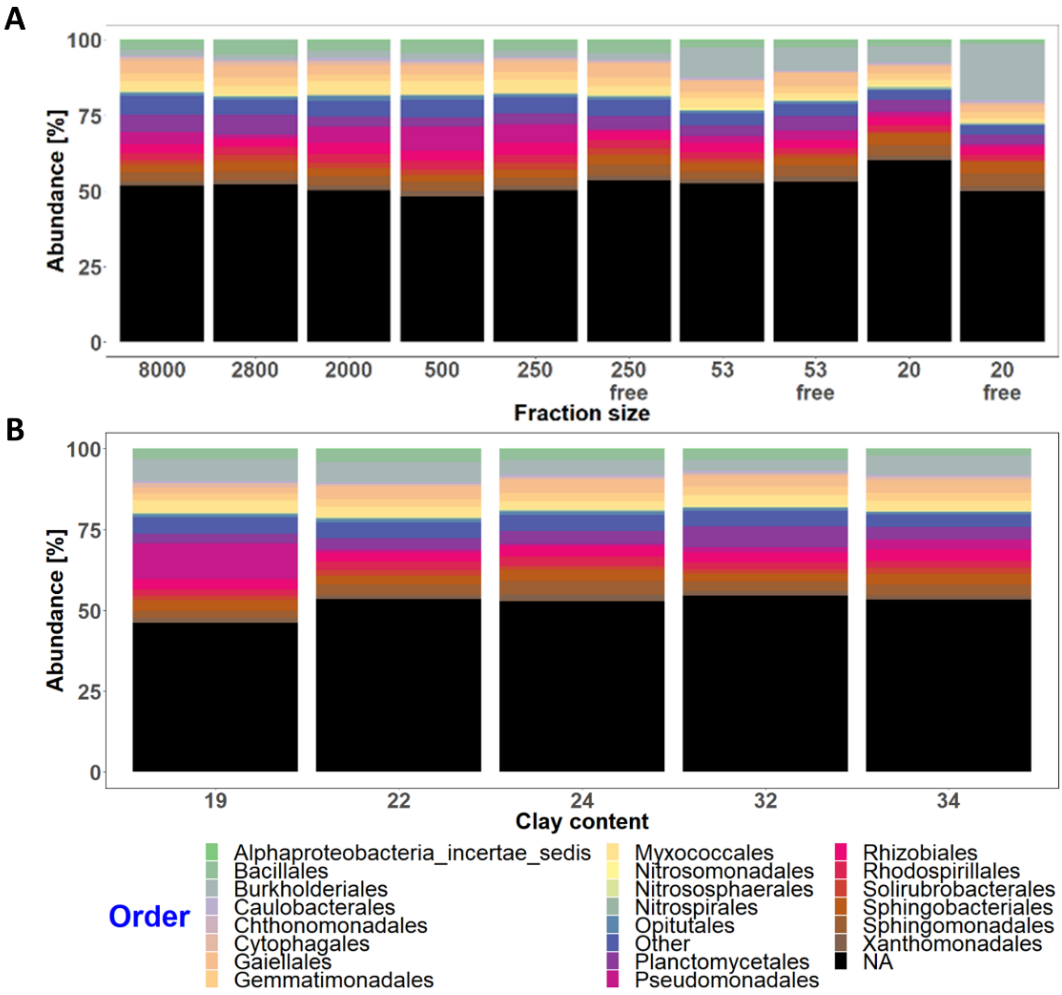
Supplementary Figure 6: Relative intensities of representative solid-state ^{13}C NMR spectra from all three clay contents and two size fractions. Normalized for alkyl-C maxima.



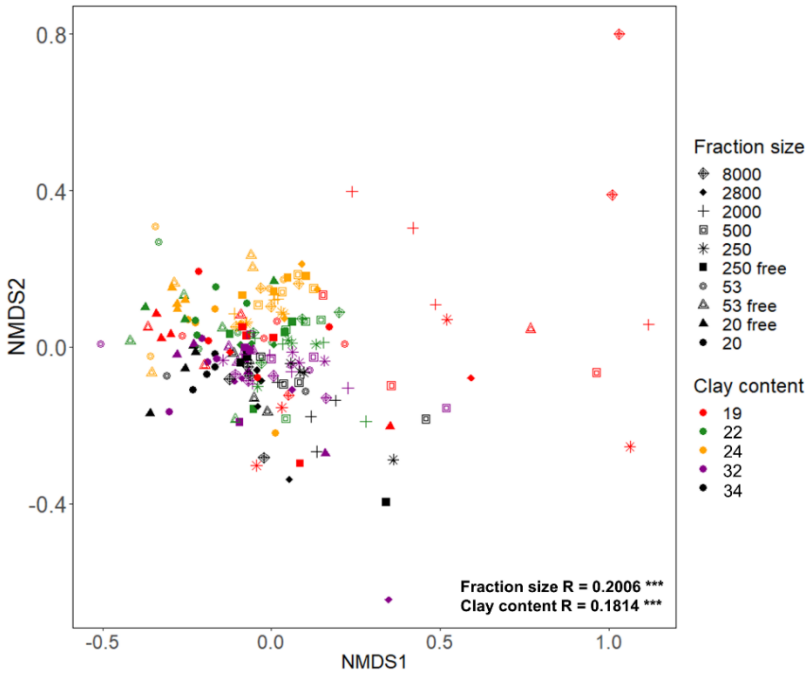
Supplementary Figure 7: NMDS plot (a) and boxplot of the alpha diversity (b) showing the negligible effect of lyophilization on bacterial diversity analysis.

VIII
Appendix B

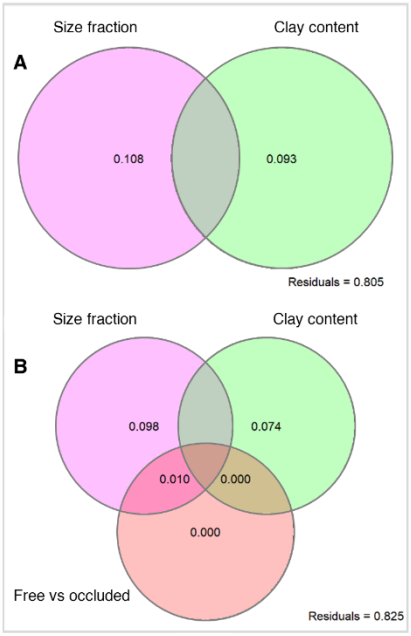
Supporting information to chapter II



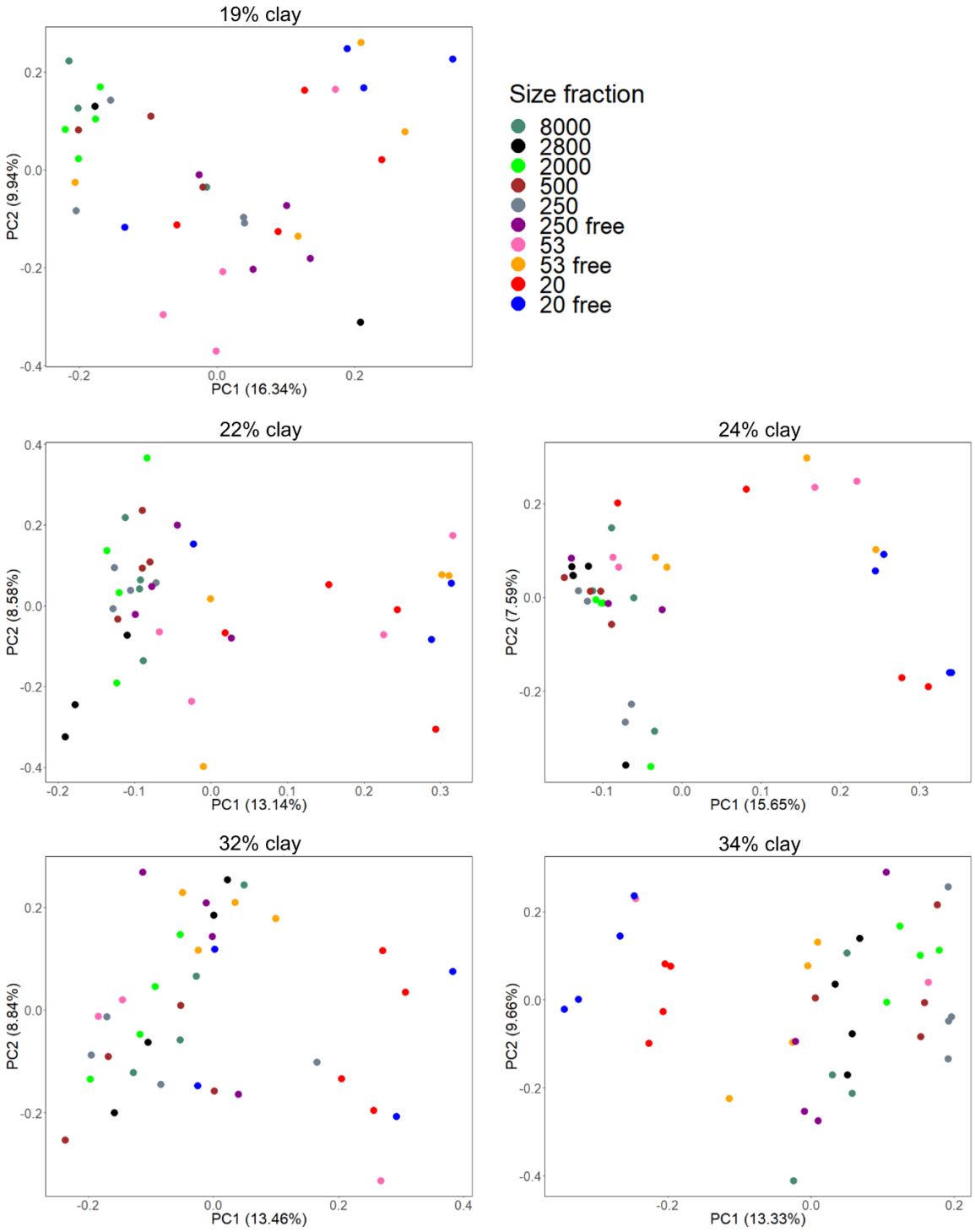
Suppl. Fig. VIII-1: Relative abundance of bacterial taxa at order level, present in soils of different size fractions (A) and clay contents (B). Orders with a mean relative abundance <0.1 are grouped as other. NAs represent sequence reads that could not be assigned to a specific order.



Suppl. Fig. VIII-2: Non-metric multidimensional scaling (NMDS) plot showing similarities in bacterial community composition between replicate samples of five clay contents and ten different aggregate size fractions. The plot reveals a slight effect of clay content and aggregate size fraction on the bacterial community composition. Statistical evaluation by analysis of similarity (ANOSIM) confirmed this by rather low but significant R-values. This plot confirms the results shown in Figure 3, whereby a few individual samples are more distinct here, but the samples of the smallest size fractions are also slightly separated here.



Suppl. Fig. VIII-3: Venn diagram showing the results of the variation partitioning analysis to assess the impact of the factors size fraction and clay content (A) as well as the impact of size fraction, clay content and location on the microaggregates (B). Empty segments had slightly negative values.



Suppl. Fig. VIII-4: Principal component analysis (PCA) showing similarities in bacterial community composition in samples from soils of different clay content. Samples are color-coded according to the different aggregate size fractions.

Supplementary tables

Suppl. Tab. VIII-1: DNA concentration in extracts after DNA extraction. Division by 10 will give recovery as µg DNA per g dry soil. *

Clay content [%]	Size fraction [µm]	Replicate 1 [ng/µl]	Replicate 2 [ng/µl]	Replicate 3 [ng/µl]	Replicate 4 [ng/µl]
19	8000	0.68	0.57	0.57	0.71
	2800	0.58	0.48	-	-
	2000	0.74	0.61	0.73	0.58
	500	0.60	0.58	0.58	0.59
	250	0.73	0.60	0.60	2.13
	250 free	1.95	1.17	1.24	1.66
	53 µm	2.12	0.86	1.13	1.02
	53 free	3.08	2.09	3.25	0.74
	< 20	20.84	26.35	25.18	19.80
	< 20 free	27.84	39.37	26.55	22.03
22	8000	1.44	4.33	1.68	3.35
	2800	1.02	2.17	1.82	2.42
	2000	2.68	2.97	2.64	2.80
	500	0.90	1.58	0.93	1.32
	250	1.31	3.00	1.50	2.21
	250 free	3.26	4.16	1.86	3.19
	53 µm	1.47	3.15	1.53	2.89
	53 free	4.29	5.13	4.12	4.02
	< 20	25.30	13.15	6.32	13.52
	< 20 free	47.18	46.10	36.71	19.87
24	8000	3.85	2.02	3.18	3.70
	2800	3.36	2.59	3.20	2.59
	2000	5.15	4.89	3.06	3.68
	500	2.56	2.58	2.08	1.56
	250	2.34	2.02	2.57	2.06
	250 free	2.58	2.50	2.36	2.50
	53 µm	3.63	2.07	2.62	2.50
	53 free	4.47	3.65	4.25	3.69
	< 20	25.61	20.19	21.20	5.23
	< 20 free	28.14	22.24	26.82	20.94
32	8000	1.70	3.48	2.14	1.82
	2800	2.30	2.20	1.38	2.32
	2000	0.74	1.53	1.83	1.77
	500	1.09	0.80	0.68	0.70
	250	0.63	1.09	1.15	0.99
	250 free	3.44	2.60	2.87	3.73
	53 µm	1.20	0.88	0.82	1.13
	53 free	2.59	1.81	2.20	1.38
	< 20	13.89	31.13	22.52	21.86
	< 20 free	12.87	14.37	19.26	16.44
34	8000	2.72	1.57	4.33	3.54

2800	1.50	1.33	1.81	4.00
2000	1.00	0.83	0.83	1.06
500	0.70	0.56	0.66	0.62
250	1.14	0.77	0.61	1.90
250 free	4.01	1.15	0.93	4.72
53 µm	0.73	0.56	0.73	0.95
53 free	1.89	1.18	2.23	2.99
< 20	28.37	24.24	22.03	34.49
< 20 free	21.68	16.47	21.33	12.63

*We used between 0.6 and 47 ng of DNA in a 10-µl PCR assay to amplify the 16S rRNA gene. At first glance it may appear that the higher amounts of DNA used in PCR for samples <20 µm may be responsible for the distinctiveness of the bacterial community composition in this aggregate size fraction. However, some samples in the <20 µm fraction with rather low DNA concentrations cluster well together with the other samples of this size fraction in PCA (Figure 3), and samples <53 µm that were similar to those of the <20 µm fraction according to PCA were not consistently those with the highest DNA concentrations. Besides, a consistent trend of increasing differences of DNA yields with increasing clay content was also not apparent, and thus not the underlying reason for the observed stronger differences in community composition with increasing clay content. In the indicator taxa analysis (Figure 4), the higher number of OTUs we identified in the smallest size fraction is also not related to higher amounts of DNA in PCR, because we worked with rarefied datasets and observed comparable alpha diversity measures (estimated richness and evenness) in all sample sets (Figure 1), indicating that these samples were not characterized by a higher diversity due to higher amounts of DNA used for PCR. Samples with the lowest DNA recovery had to some extent, though not consistently, the lowest alpha diversity values. Thus, we are convinced that diversity is not systematically over- or underestimated due to variable amounts of DNA input material.

Suppl. Tab. VIII-2: Results of the indicator taxa analysis. Presented is the indicator value (IV) and the relative abundance of each taxon that was identified as being characteristic for a specific aggregate size fraction. All taxa listed here have a Benjamini and Hochberg adjusted p-value <0.05.

Size Fraction	Phylum	Class	Genus	Order	Family	Species	IV	Rel. abund.
8000	*Proteobacteria*	*Gammaproteobacteria*	NA	NA	NA	NA	0.443	0.016
2800	*Firmicutes*	*Bacilli*	*Bacillus*	*Bacillales*	*Bacillaceae*_1	*Bacillus muralis*	0.423	0.502
2800	*Firmicutes*	*Clostridia*	*Clostridium* s. str.	*Clostridiales*	*Clostridiaceae*_1	NA	0.442	0.008
	Proteobacteria	NA	NA	NA	NA	NA	0.410	0.005
	Proteobacteria	*Alphaproteobacteria*	NA	*Rhodospirillales*	NA	NA	0.469	0.013
2000	*Bacteroidetes*	*Sphingobacteriia*	*Terrimonas*	*Sphingobacteriales*	*Chitinophagaceae*	*Terrimonas arctica*	0.478	0.022
	Bacteroidetes	*Cytophagia*	NA	*Cytophagales*	NA	NA	0.438	0.246
	Bacteroidetes	*Sphingobacteriia*	NA	*Sphingobacteriales*	*Chitinophagaceae*	NA	0.455	0.044
	NA	NA	NA	NA	NA	NA	0.421	0.082
	NA	NA	NA	NA	NA	NA	0.479	0.032
	Proteobacteria	*Gammaproteobacteria*	NA	NA	NA	NA	0.478	0.373
	Proteobacteria	*Deltaproteobacteria*	NA	*Myxococcales*	NA	NA	0.437	0.038
	Proteobacteria	*Gammaproteobacteria*	NA	NA	NA	NA	0.505	0.040
	Proteobacteria	*Alphaproteobacteria*	NA	*Caulobacterales*	*Caulobacteraceae*	NA	0.419	0.118
	Proteobacteria	*Deltaproteobacteria*	NA	*Myxococcales*	NA	NA	0.543	0.114
	Proteobacteria	*Alphaproteobacteria*	NA	NA	NA	NA	0.453	0.055
	Proteobacteria	*Alphaproteobacteria*	*Pseudolabrys*	*Rhizobiales*	*Xanthobacteraceae*	*Pseudolabrys taiwanensis*	0.433	0.044
	Proteobacteria	*Alphaproteobacteria*	*Dongia*	*Rhodospirillales*	*Rhodospirillaceae*	*Dongia rigui*	0.522	0.069
	Proteobacteria	*Alphaproteobacteria*	NA	*Rhodospirillales*	*Rhodospirillaceae*	NA	0.436	0.061
	Proteobacteria	*Deltaproteobacteria*	NA	*Myxococcales*	NA	NA	0.535	0.062
	Proteobacteria	*Gammaproteobacteria*	*Steroidobacter*	*Xanthomonadales*	*Sinobacteraceae*	NA	0.460	0.047
	Proteobacteria	*Gammaproteobacteria*	*Dokdonella*	*Xanthomonadales*	*Xanthomonadaceae*	NA	0.506	0.053
	Proteobacteria	*Alphaproteobacteria*	*Dongia*	*Rhodospirillales*	*Rhodospirillaceae*	NA	0.557	0.058
	Proteobacteria	*Alphaproteobacteria*	NA	NA	NA	NA	0.425	0.098

Size Fraction	Phylum	Class	Genus	Order	Family	Species	IV	Rel. abund.
	Proteobacteria	*Deltaproteobacteria*	NA	*Myxococcales*	NA	NA	0.466	0.299
	Proteobacteria	*Gammaproteobacteria*	*Dokdonella*	*Xanthomonadales*	*Xanthomonadaceae*	NA	0.411	0.135
2000	*Verrucomicrobia*	*Opitutae*	NA	*Opitutales*	*Opitutaceae*	NA	0.481	0.193
500	NA	NA	NA	NA	NA	NA	0.411	0.006
500	*Proteobacteria*	NA	NA	NA	NA	NA	0.443	0.013
	Proteobacteria	*Deltaproteobacteria*	NA	*Myxococcales*	NA	NA	0.482	0.057
	Proteobacteria	*Deltaproteobacteria*	NA	NA	NA	NA	0.462	0.017
	Proteobacteria	*Betaproteobacteria*	NA	NA	NA	NA	0.447	0.023
	Verrucomicrobia	Subdivision3	Subdivision3 inc. sed.	NA	NA	NA	0.404	0.228
250	*Actinobacteria*	*Actinobacteria*	*Solirubrobacter*	*Solirubrobacterales*	*Solirubrobacteraceae*	NA	0.425	0.533
	NA	NA	NA	NA	NA	NA	0.517	0.006
	NA	NA	NA	NA	NA	NA	0.439	0.158
	NA	NA	NA	NA	NA	NA	0.431	0.012
	Proteobacteria	*Deltaproteobacteria*	NA	*Myxococcales*	NA	NA	0.455	0.569
	Proteobacteria	*Alphaproteobacteria*	NA	*Rhizobiales*	NA	NA	0.397	0.220
250 free	*Gemmatimonade-tes*	*Gemmatimonadetes*	*Gemmatimonas*	*Gemmatimonadales*	*Gemmatimonadaceae*	*Gemmatimonas auranti-aca*	0.416	0.003
53	*Proteobacteria*	*Betaproteobacteria*	*Nitrosospira*	*Nitrosomonadales*	*Nitrosomonadaceae*	NA	0.461	0.211
53 free	*Planctomycetes*	*Planctomycetia*	NA	*Planctomycetales*	*Planctomycetaceae*	NA	0.409	0.002
	Verrucomicrobia	*Spartobacteria*	NA	NA	NA	NA	0.447	0.001
<20	*Acidobacteria*	*Acidobacteria*_Gp6	Gp6	NA	NA	NA	0.440	0.050
	Acidobacteria	*Acidobacteria*_Gp6	Gp6	NA	NA	NA	0.427	0.106
	Acidobacteria	*Acidobacteria*_Gp6	Gp6	NA	NA	NA	0.380	0.629
	Acidobacteria	*Acidobacteria*_Gp4	Gp4	NA	NA	NA	0.505	0.232
	Acidobacteria	*Acidobacteria*_Gp4	Gp4	NA	NA	NA	0.374	0.003
	Acidobacteria	*Acidobacteria*_Gp3	Gp3	NA	NA	NA	0.484	0.133
	Acidobacteria	*Acidobacteria*_Gp7	Gp7	NA	NA	NA	0.457	0.094
	Acidobacteria	*Acidobacteria*_Gp4	Gp4	NA	NA	NA	0.522	0.068
	Acidobacteria	*Acidobacteria*_Gp3	Gp3	NA	NA	NA	0.431	0.084

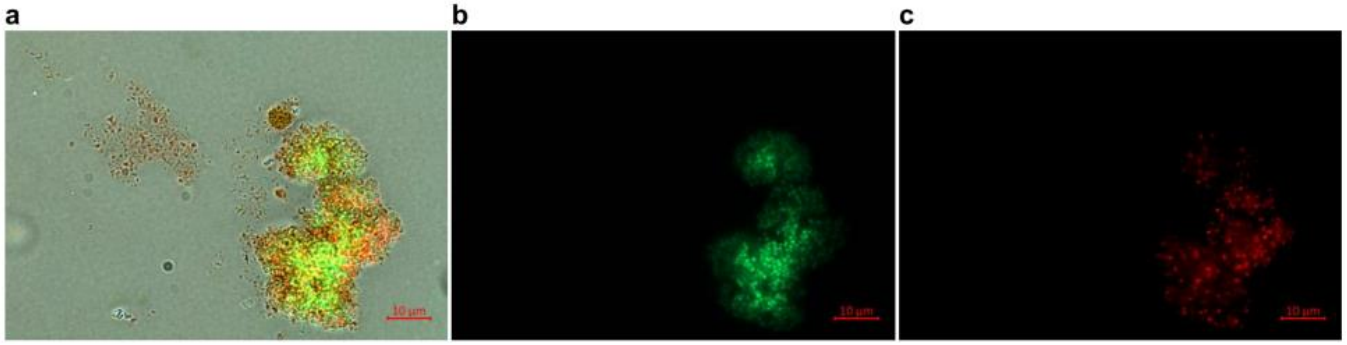
Size Fraction	Phylum	Class	Genus	Order	Family	Species	IV	Rel. abund.
	Acidobacteria	*Acidobacteria*_Gp6	Gp6	NA	NA	NA	0.416	0.580
	Acidobacteria	*Acidobacteria*_Gp4	NA	NA	NA	NA	0.425	0.036
<20	*Acidobacteria*	*Acidobacteria*_Gp4	*Aridibacter*	NA	NA	*Aridibacter kavangonen-sis*	0.467	0.010
	Acidobacteria	*Acidobacteria*_Gp4	NA	NA	NA	NA	0.450	0.010
	Acidobacteria	*Acidobacteria*_Gp4	Gp4	NA	NA	NA	0.473	0.315
	Acidobacteria	*Acidobacteria*_Gp3	Gp3	NA	NA	NA	0.412	0.093
	Acidobacteria	*Acidobacteria*_Gp4	Gp4	NA	NA	NA	0.476	0.071
	Acidobacteria	*Acidobacteria*_Gp4	NA	NA	NA	NA	0.430	0.015
	Acidobacteria	*Acidobacteria*_Gp6	Gp6	NA	NA	NA	0.419	0.128
	Acidobacteria	*Acidobacteria*_Gp7	Gp7	NA	NA	NA	0.493	0.131
	Actinobacteria	*Actinobacteria*	NA	NA	NA	NA	0.454	0.022
	Bacteroidetes	*Flavobacteriia*	*Flavobacterium*	*Flavobacteriales*	*Flavobacteriaceae*	NA	0.485	0.020
	Bacteroidetes	*Sphingobacteriia*	NA	*Sphingobacteriales*	*Chitinophagaceae*	NA	0.418	0.174
	Bacteroidetes	NA	NA	NA	NA	NA	0.474	0.031
	Bacteroidetes	*Sphingobacteriia*	NA	*Sphingobacteriales*	*Chitinophagaceae*	NA	0.457	0.177
	Bacteroidetes	*Sphingobacteriia*	*Terrimonas*	*Sphingobacteriales*	*Chitinophagaceae*	NA	0.475	0.183
	CD WPS-1	NA	NA	NA	NA	NA	0.476	0.018
	CD WPS-1	NA	NA	NA	NA	NA	0.456	0.218
	CD WPS-1	NA	NA	NA	NA	NA	0.445	0.021
	CD WPS-1	NA	NA	NA	NA	NA	0.446	0.055
	Gemmatimonade-tes	*Gemmatimonadetes*	*Gemmatimonas*	*Gemmatimonadales*	*Gemmatimonadaceae*	*Gemmatimonas aranti-aca*	0.414	0.115
	Gemmatimonade-tes	*Gemmatimonadetes*	*Gemmatimonas*	*Gemmatimonadales*	*Gemmatimonadaceae*	*Gemmatimonas aranti-aca*	0.402	0.083
	Latescibacteria	NA	NA	NA	NA	NA	0.477	0.063
	Latescibacteria	NA	NA	NA	NA	NA	0.453	0.053
	NA	NA	NA	NA	NA	NA	0.499	0.008
	NA	NA	NA	NA	NA	NA	0.542	0.061
	NA	NA	NA	NA	NA	NA	0.456	0.078

Size Fraction	Phylum	Class	Genus	Order	Family	Species	IV	Rel. abund.
	NA	NA	NA	NA	NA	NA	0.467	0.040
	NA	NA	NA	NA	NA	NA	0.451	0.011
<20	NA	NA	NA	NA	NA	NA	0.411	0.201
	NA	NA	NA	NA	NA	NA	0.482	0.044
	NA	NA	NA	NA	NA	NA	0.436	0.109
	Nitrospirae	*Nitrospira*	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	NA	0.452	0.076
	Proteobacteria	*Betaproteobacteria*	NA	NA	NA	NA	0.425	0.086
	Verrucomicrobia	*Spartobacteria*	*Spartobacteria* inc. sed.	NA	NA	NA	0.461	0.127
	Verrucomicrobia	*Spartobacteria*	*Spartobacteria* inc. sed.	NA	NA	NA	0.415	0.242
<20 free	*Acidobacteria*	*Acidobacteria*_Gp4	NA	NA	NA	NA	0.468	0.118
	Acidobacteria	*Acidobacteria*_Gp4	NA	NA	NA	NA	0.504	0.051
	Acidobacteria	*Acidobacteria*_Gp4	*Aridibacter*	NA	NA	NA	0.504	0.018
	Acidobacteria	*Acidobacteria*_Gp4	NA	NA	NA	NA	0.560	0.059
	Acidobacteria	*Acidobacteria*_Gp4	NA	NA	NA	NA	0.539	0.359
	Acidobacteria	*Acidobacteria*_Gp6	Gp6	NA	NA	NA	0.457	0.023
	Actinobacteria	*Actinobacteria*	*Aeromicrobium*	*Actinomycetales*	*Nocardioidaceae*	NA	0.560	0.006
	Armatimonadetes	*Chthonomonadetes*	*Chthonomonas*	*Chthonomonadales*	*Chthonomonadaceae*	NA	0.443	0.016
	Bacteroidetes	*Sphingobacteriia*	NA	*Sphingobacteriales*	*Chitinophagaceae*	NA	0.478	0.124
	Bacteroidetes	*Sphingobacteriia*	*Pedobacter*	*Sphingobacteriales*	*Sphingobacteriaceae*	NA	0.451	0.007
	Bacteroidetes	*Sphingobacteriia*	*Flavisolibacter*	*Sphingobacteriales*	*Chitinophagaceae*	*Flavisolibacter ginsen-giterrae*	0.444	0.123
	Bacteroidetes	NA	NA	NA	NA	NA	0.527	0.001
	Bacteroidetes	*Sphingobacteriia*	NA	*Sphingobacteriales*	*Sphingobacteriaceae*	NA	0.493	0.091
	Bacteroidetes	*Sphingobacteriia*	*Mucilaginibacter*	*Sphingobacteriales*	*Sphingobacteriaceae*	NA	0.480	0.012
	Bacteroidetes	*Sphingobacteriia*	*Mucilaginibacter*	*Sphingobacteriales*	*Sphingobacteriaceae*	*Mucilaginibacter rigui*	0.548	0.011
	Can. Saccharibac-teria	NA	Saccharibacteria inc. sed.	NA	NA	NA	0.411	0.001
	Can. Saccharibac-teria	NA	NA	NA	NA	NA	0.429	0.001
	CD WPS-1	NA	NA	NA	NA	NA	0.427	0.005

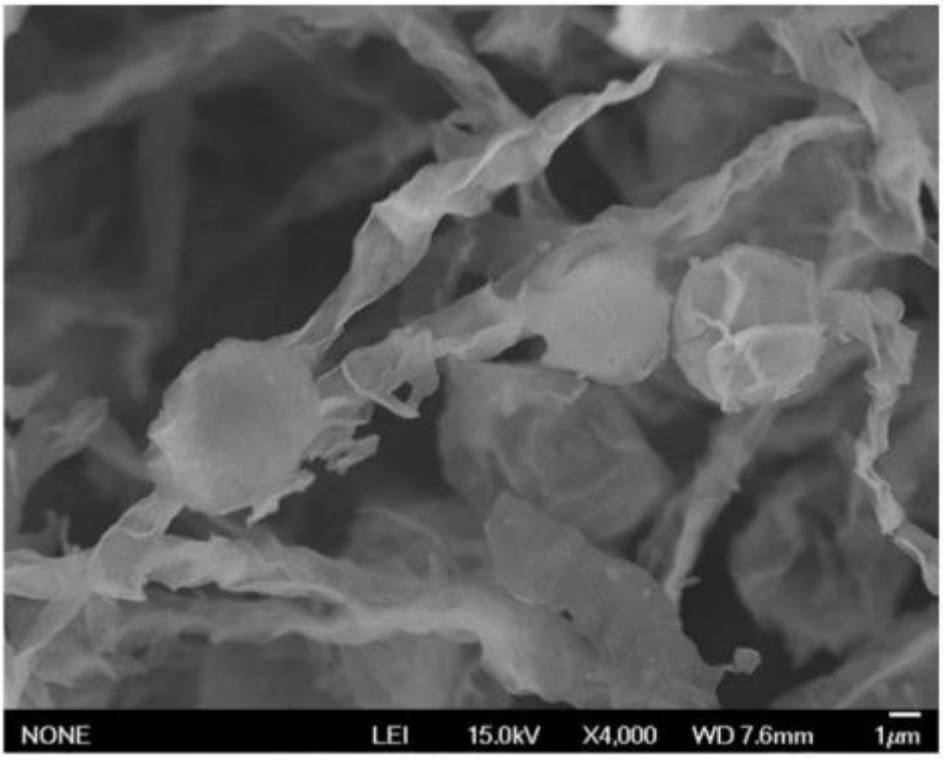
Size Fraction	Phylum	Class	Genus	Order	Family	Species	IV	Rel. abund.
<20 free	NA	NA	NA	NA	NA	NA	0.547	0.007
	NA	NA	NA	NA	NA	NA	0.424	0.049
	NA	NA	NA	NA	NA	NA	0.610	0.040
	NA	NA	NA	NA	NA	NA	0.462	0.100
	NA	NA	NA	NA	NA	NA	0.458	0.156
	NA	NA	NA	NA	NA	NA	0.582	0.019
	NA	NA	NA	NA	NA	NA	0.475	0.013
	Proteobacteria	*Betaproteobacteria*	*Aquabacterium*	*Burkholderiales*	*Burkholderiales inc. sed*	*Aquabacterium citratiphi-lum*	0.426	0.181
	Proteobacteria	*Alphaproteobacteria*	*Novosphingobium*	*Sphingomonadales*	*Sphingomonadaceae*	NA	0.530	0.011
	Proteobacteria	*Alphaproteobacteria*	*Sphingopyxis*	*Sphingomonadales*	*Sphingomonadaceae*	NA	0.517	0.005
	Proteobacteria	*Alphaproteobacteria*	NA	*Alphaproteobacteria* inc. sed	NA	NA	0.401	0.211
	Proteobacteria	*Betaproteobacteria*	NA	*Methylophilales*	*Methylophilaceae*	NA	0.556	0.005
	Proteobacteria	*Gammaproteobacteria*	NA	*Xanthomonadales*	*Xanthomonadaceae*	NA	0.513	0.039
	Proteobacteria	*Alphaproteobacteria*	*Caulobacter*	*Caulobacterales*	*Caulobacteraceae*	*Caulobacter segnis*	0.532	0.092
	Proteobacteria	*Betaproteobacteria*	*Aquabacterium*	*Burkholderiales*	*Burkholderiales* inc. sed	*Aquabacterium commune*	0.672	0.083
	Proteobacteria	*Betaproteobacteria*	NA	*Burkholderiales*	NA	NA	0.678	0.028
	Proteobacteria	*Alphaproteobacteria*	*Mesorhizobium*	*Rhizobiales*	*Phyllobacteriaceae*	*Mesorhizobium loti*	0.464	0.026
	Proteobacteria	*Gammaproteobacteria*	NA	*Pseudomonadales*	*Moraxellaceae*	NA	0.592	0.021
	Proteobacteria	*Gammaproteobacteria*	NA	NA	NA	NA	0.447	0.001
	Proteobacteria	*Betaproteobacteria*	NA	*Burkholderiales*	NA	NA	0.629	1.840
	Proteobacteria	*Betaproteobacteria*	*Ralstonia*	*Burkholderiales*	*Burkholderiaceae*	*Ralstonia pickettii*	0.668	0.733
	Proteobacteria	*Alphaproteobacteria*	*Sphingomonas*	*Sphingomonadales*	*Sphingomonadaceae*	NA	0.502	0.196
	Proteobacteria	*Gammaproteobacteria*	*Aquicella*	*Legionellales*	*Coxiellaceae*	NA	0.453	0.003

IX
Appendix C

Supporting information to chapter III



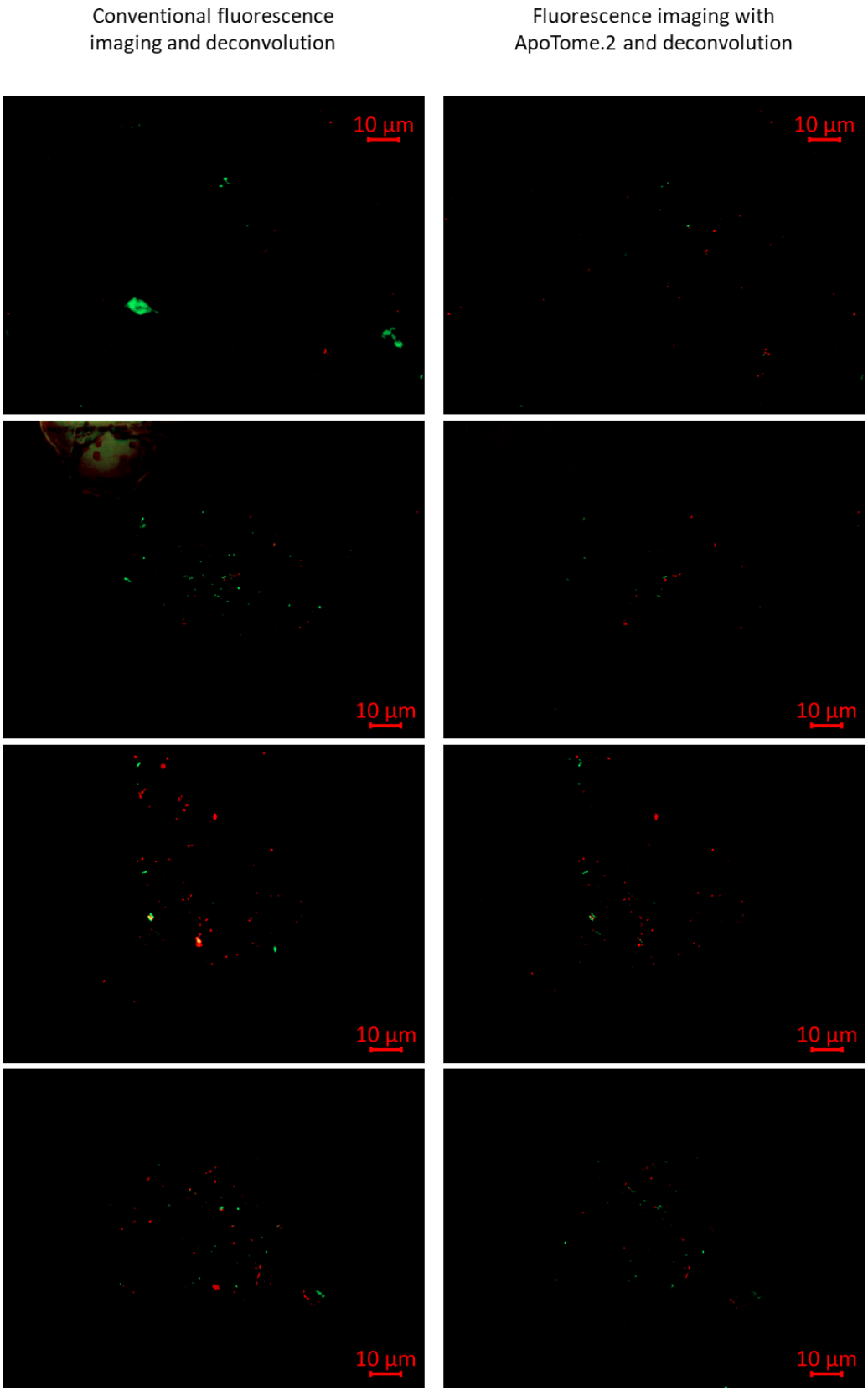
Suppl. Fig. IX-1: Viability stains of *Gordonia alkanivorans* showing the brightfield and fluorescence image (a) with viable cells in the green (495 nm) channel (b) and non-viable cells in the red channel (570 nm) (b).



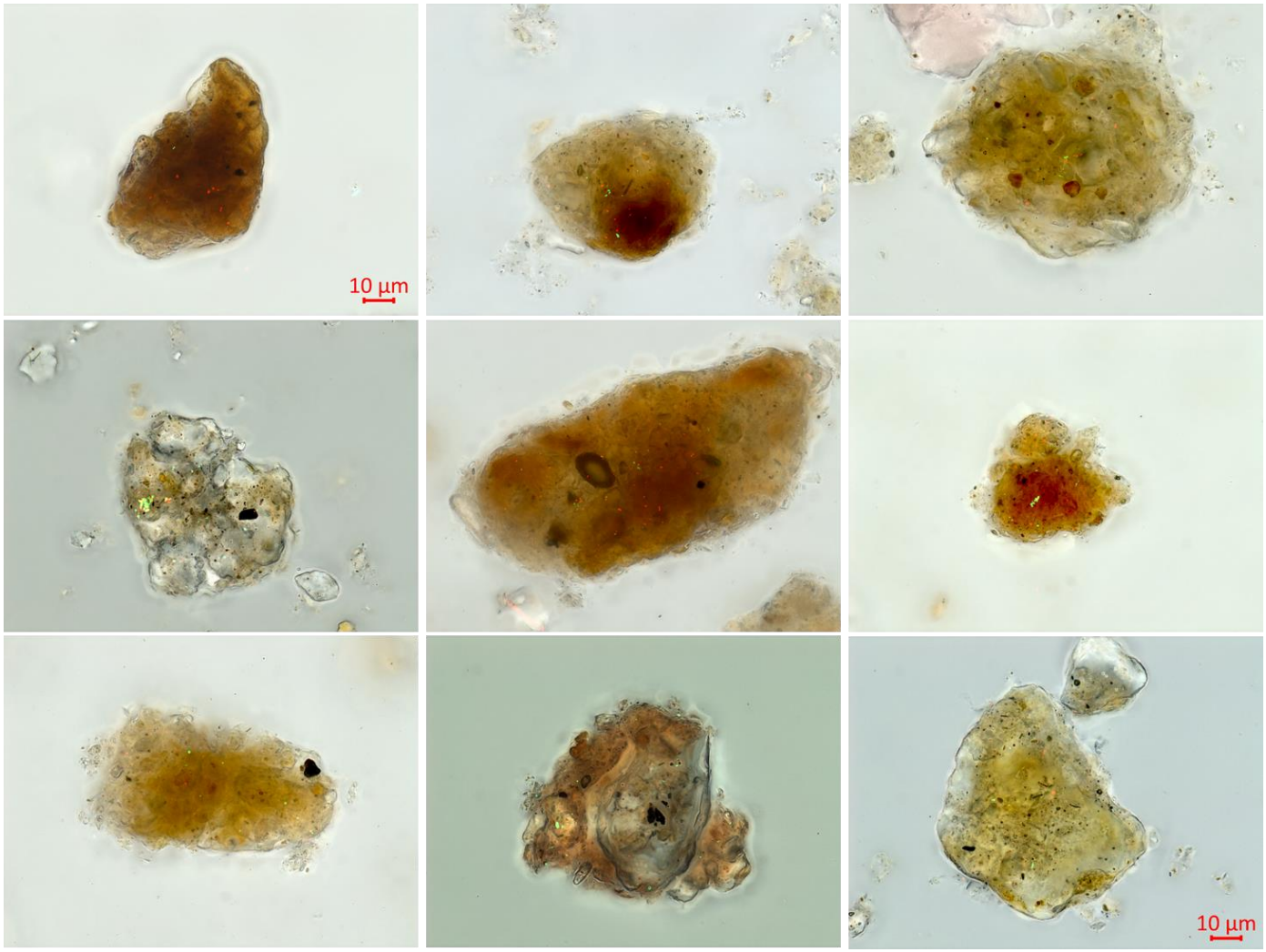
Suppl. Fig. IX-2: SEM micrograph of *P. protegens* wildtype at t_3 incubated with montmorillonite and goethite receiving the wet-dry cycles showing microbial cell envelopes and the disrupted cell walls.

X
Appendix D

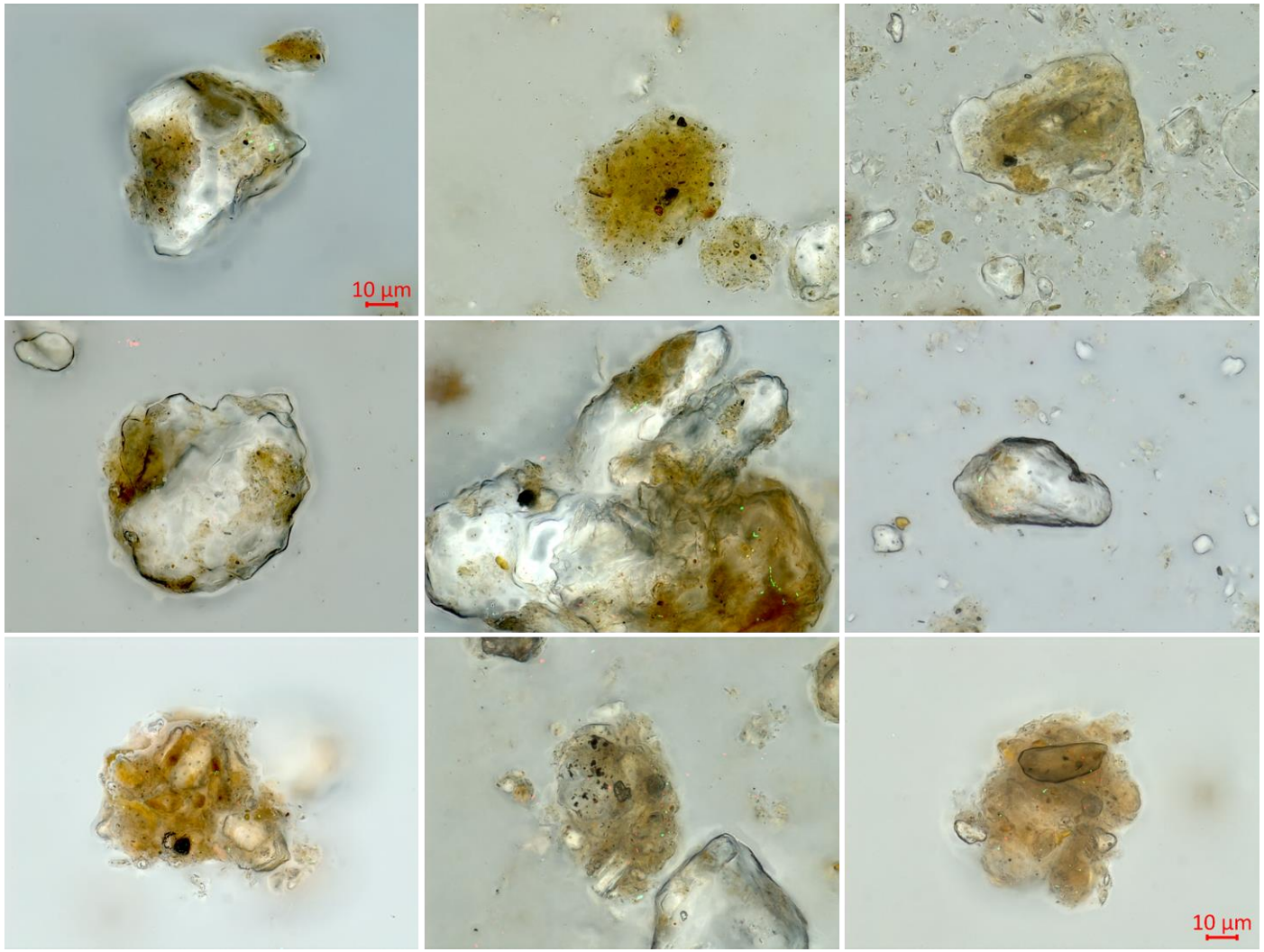
Supporting information to chapter IV



Suppl. Fig. X-1: Comparison of the captured fluorescence signals with and without the use of the ApoTome



Suppl. Fig. X-2: Morphological and compositional heterogeneity as well as variation in microbial colonization of further individual microaggregates. Green fluorescent signals represent vital and red fluorescent signals dead bacterial cells. The images were obtained by fluorescence imaging using the ApoTome and subsequent deconvolution.



Suppl. Fig. X-3: Morphological and compositional heterogeneity as well as variation in microbial colonization of further individual microaggregates. Green fluorescent signals represent vital and red fluorescent signals dead bacterial cells. The images were obtained by fluorescence imaging using the ApoTome and subsequent deconvolution.

Description of the experimental setup the aggregate soil samples were taken from

Excerpt modified on the basis of the manuscript:

Amelung, W.; Meyer, N.; Rodionov, A.; Aehnelt, M.; Bauke, S.;Biesgen, D.; Dultz, S.; Guggenberger, G.; Jaber, M.; Knief, C.; Klumpp, E.; Kögel-Knabner, I.; Nischwitz, V.; Schweizer, S.; Wu, B.; Totsche, K.U.; Lehndorff, E., 2021. Process sequence of aggregate formation as disentangled through multi-isotope labelling. Geoderma

Manuscript submitted

Materials and Methods

To understand processes of initial soil aggregate formation and turnover, a microcosm study was conducted in a climate chamber, using isotope-labelled organic and inorganic model compounds (^{13}C-EPS, ^{15}N-bacteria, ^{57}Fe-FeOOH, and ^{29}Si-montmorillonite) as major constituents of soil aggregate formation (Fig S1, Supplementary Materials, see online version).

For the production of ^{13}C-labelled EPS, we used *Bacillus subtilis* strain 168 [DSM 402], and employed a harvesting protocol presented as by Omoike and Chorover (2006). The bacterial cells were streaked onto fresh ^{12}C-glucose agar plates (glucose medium), cultured for 16 h under aerobic conditions, harvested, added to 50 ml ^{12}C glucose liquid medium, and were again allowed to grow overnight. Around 1-2 mL of the culture was transferred to 50 mL fresh ^{13}C-glucose medium and allowed to grow for 24 h. From this, again, 1-2 mL of the culture was added to new ^{13}C glucose medium (24 h of growth). This step was repeated up to four times to increase the incorporation of the ^{13}C-label. The cultures were harvested after 24 h of growth, i.e., in the stationary growth phase, when visibly maximum aggregation and viscosity occurred. EPS was separated from the cells by centrifugation (10,000 x g, 4 °C). The pelleted cells were re-dispersed twice in the same supernatant; the final centrifugation step lasted 40 minutes. Finally, a volume reduction of the supernatant by 90% was achieved using freeze-drying. The concentrated EPS was precipitated in cold ethanol (70% of final concentration) for 12 h, and the precipitate was separated from the ethanol suspension by centrifugation (12,000 x g, 30 min at 4 °C) and re-suspended in ultrapure (Milli-Q) water. This extraction was repeated 3 times (to obtain further precipitation of EPS in the supernatant). The pellet obtained after centrifugation was dissolved in Milli-Q water and dialyzed against the Milli-Q water using Spectra/Por 7 regenerated cellulose (RC) membranes (1000 MWCO from Spectrum Europe, Breda, NL) to remove ethanol and entrained media residues. After dialysis for 72 h against two changes of Milli-Q water per day, the solution was freeze-dried to a dry, fibrous matrix and stored at -20 °C prior to use. Overall, ^{13}C-EPS was extracted with a mass yield of up to 400 mg L^{-1} of labelled EPS, which is in good agreement with Omoike and Chorover (2006) and Decho and Moriarty (1990), reporting yields of 420 mg $L^{-1}$and up to 3 g L^{-1}, respectively. Samples never exceeded 4°C during the whole extraction procedure.

To obtain ^{15}N-labelled bacterial biomass, four different strains with specific characteristics were selected as model organisms to reflect soil bacterial diversity: i) *Pseudomonas prote-*

gens CHA0 gfp2 (Péchy-Tarr et al., 2013), a green fluorescence protein (GFP) labeled mutant of *Pseudomonas protegens* CHA0, which is a widely distributed, fast-growing soil bacterium with a broad substrate spectrum, ii) *Methylocystis parvus* DWT (Knief & Dunfield, 2005), a slow-growing soil bacterium that utilizes methane as its sole carbon and energy source, iii) *Streptomyces viridosporus* (DSM 40243^T) (Pridham et al., 1958), a soil bacterium characterized by its filamentous growth characteristics and its ability to degrade complex and hard-to-degrade carbon compounds such as lignin, and iv) *Gordonia alkanivorans* (DSM 44187) (Kummer et al., 1999), a soil bacterium characterized by its hydrophobic cell surface. *P. protegens* CHA0 gfp2 was cultivated in liquid culture on a shaker at 260 rpm in M9 medium (DSMZ medium 382) with the addition of gentamycin. *M. parvus* DWT was cultured on AMS medium agar plates under a methane atmosphere (ATCC medium 784). *S. viridosporus* was cultured on a shaker at 320 rpm in ISP4 liquid medium (DSMZ medium 547). *G. alkanivorans* was cultured on MSM solid medium (Rhee et al., 1998). All bacterial cultures were grown at 30°C. The ^{15}N labelling of bacterial cells was performed in AMS, MSM and M9 media by the addition of $^{15}NH_4Cl$ (≥98 atom % ^{15}N) (Sigma-Aldrich, St. Louis, USA) and in ISP4 medium by the addition of $(^{15}NH_4)_2SO_4$ (98 atom % ^{15}N) (Sigma-Aldrich, St. Louis, USA). On the starting day of the microcosm experiment, the bacterial cultures were harvested, re-suspended in sterile water and pooled. Due to its filamentous growth, the *S. viridosporus* culture was passed through a 150 µm mesh before being added to the final suspension. In the final cultures 50.4 % of bacterial N was ^{15}N (Tab. X-1).

Suppl. Tab. X-1: N from Living bacteria, C from extracellular polymeric substances (EPS), Fe and clay labels applied to homogenized Ap horizon from a Haplic Luvisol. Three gauze bags per pot filled with labeled soil (3x13 g per pot; total labeled pots = 2x3x3).

	Added amount [g kg-1]	Labeled representative	Isotope enrichment
Living bacteria	27.75[1]	^{15}N- *Pseudomonas protegens* ^{15}N- *Methylocystis pavus* ^{15}N-*Gordonia alkanivorans* ^{15}N- *Streptomyces viridosporus*	54.3 % ^{15}N of 1.082 g added N[2]
EPS	1.22	^{13}C-EPS	3.7 % ^{13}C of 0.57 g added C
Oxide	0.456	$^{57}FeOOH*0.4H_2O$	95.5 % ^{57}Fe of 0.25 g added Fe
Clay	25.03	^{29}Si-montmorillonite	10 % ^{29}Si of 6.5 g added Sl

[1] fresh weight bacterial biomass
[2] calculation based on 13% N in dry bacterial biomass and 70% water content of fresh bacterial biomass

For synthesis of ^{57}Fe labelled goethite, powdered ^{57}Fe metal with a degree of enrichment >95.5 % was obtained from EURISO-TOP (Saint Aubin, France). The Fe powder was dissolved in concentrated H_2SO_4 p.a., and Fe was oxidized to the trivalent form by addition of 30% H_2O_2 for trace analysis (chemicals from VWR International GmbH, Darmstadt, Germany). Synthesis of goethite started by addition of 10 M NaOH under continuous stirring up to pH 12. The suspension was kept at 25°C for 13 days (Cambier, 1986). After adjusting to pH 6 by addition of 0.1 M HCl the goethite was washed with deionized water by centrifugation and decantation until the electrical conductivity was <20 µS cm^{-1}. Finally, the suspension was freeze-dried. The goethite crystallites showed needle-like habitus with an average needle length of 0.5 µm and an average thickness of 0.03 µm as determined by scanning electron microscopy (SEM; FEI Quanta 200, Hillsboro, USA) (Fig. S2a; Supplementary Materials, see online version). The purity of the goethite produced was verified by X-ray diffraction (XRD; CuKα radiation; 40 kV, 30 mA) using a Siemens D500 (Karlsruhe, Germany) instrument (Fig. S2b, Supplementary Materials, see online version). The specific surface area (SSA) measured by N_2 adsorption at 77 K (Quantachrome Nova 4000e, Boynton Beach, USA) was 75 ± 5 m^2 g^{-1}.

The ^{29}Si-labelled montmorillonite, $Ca_{0.2}(Al_{1.6}Mg_{0.4})[Si_4O_{10}(OH,F)_2]$, was synthesized according to the standard procedure for clay minerals outlined by Jaber et al. (2012) and Jacquemot et al. (2019), using 100% of ^{29}Si labelled tetraethoxysilane as silica source. The hydrogel was obtained by adding 25.1 mL of a solution of aluminium nitrate (81.2 g L^{-1}) and 4.01 mL of a solution of magnesium nitrate (100.45 g L^{-1}) into a 15 mL solution of ethanol containing 0.1 mol of labelled TEOS (tetraethylorthosilicate) and 0.005 mol of $CaCO_3$. An ammoniacal solution (33%) was then added until gelification. The gel was stored one night at room temperature and heated one day at 200°C and another day at 650°C. The dried "gel" was then transferred into an internal silver tubing placed in an externally-heated Morey-type pressure vessel. Synthesis conditions were a temperature of 400°C, a water pressure of 1 kbar, and a duration of four weeks. The final product had a ^{29}Si label of about 10% (Tab. X-1), with a layer density of d001 = 1.4 nm, as typical for 3-layer clay minerals (Figure S3a, Supplementary Materials, see online version). The sharp d001 basal spacing evidences significant size and crystallinity; transmission electron microscopy (TEM) confirms voluminous structure prior to manual grinding (Figure S3b, Supplementary Materials, see online version). After synthesis, the EPS, goethite, and montomorillonite were freeze dried, sieved <250 µm and added in

equal amounts to dry-sieved microaggregates (<250 µm) from a Stagnic Luvisol derived from loess (John et al., 2005) and collected near Rotthalmünster, Germany, located in the Tertiary mountainous area (Tertiäres Hügelland) in the lower Rottal area. The soil had 17% clay (< 2 µm), 30% fine silt (2-20 µm), 43% coarse silt (20-63 µm) and 10% sand (63-2000 µm) in the Ap horizon; dominant minerals of the clay fraction were illite > kaolinite > vermiculite (John et al., 2005). Dry-sieving of the Ap soil sample to <250 µm was done to pre-destroy macroaggregates, as, e.g., also suggested by DeGryze et al. (2006) for aggregation experiments. Thereafter, the ^{15}N bacterial suspension was added with a spray bottle while constantly mixing the soil substrate at a water content of 20% w/w. Table 1 provides details on the added amounts. The spiked soil was then filled into gauze bags with 13 g each and three bags were placed in the topmost 10 cm of a pot. The gauze (mesh size 1 mm) was selected to hinder coarse roots but to allow fine roots and hyphae to enter the bags and support aggregate formation. Each treatment consisted of three pots. We thus prepared 18 pots, 9 of which were planted with *Festuca heteromalla* as a model plant (1 seed cm^{-2}). Additionally, 9 plant-free pots representing bare soil and additionally a total of 18 corresponding pots with unlabeled soils served as controls (see Fig. S1; Supplementary Materials for experiment details). All pots were incubated in a climate chamber under environmental conditions typical for summer in Germany (14 h day-time with 25°C, 10 h night-time with 15°C, 450 µmol m^{-2} s^{-1} light intensity, 70% humidity). Artificial drip irrigation then continued in weekly intervals in order to prevent the pots from drying and to maintain soil moisture at 20 ± 3% (w/w).

Festuca has a dense root growth and is resistant against nutrient (N) stress. Root growth was controlled by cutting the above ground plant biomass (Volder et al., 2007), to about 10 cm after sampling at 4 and 12 weeks to prevent the formation of dense root balls in the pots that would complicate obtaining complete macro- and microaggregates even when protected from coarse roots in gauze bags. Soil samples were taken after 4 and 12 weeks of incubation and after one complete vegetation period of 30 weeks.

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