Emerging threats to the stability of soil aggregates

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YUN LIANG

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1st Reviewer: Prof. Dr. Matthias C. Rillig
2nd Reviewer: Prof. Dr. Britta Tietjen

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Declaration

I am here to declare I finished the dissertation independently. The dissertation was reviewed by Prof. Dr. Matthias C. Rillig and Prof. Dr. Britta Tietjen, the defense was conducted on 25.05.2021 at 10:45.

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SUMMARY

Soil aggregates are clusters of soil particles, supporting the soil physical structure. Soil aggregation is key to the functions of ecosystem, including aeration, water infiltration and storage, nutrient cycling, and the pore system.

This doctoral work investigates the effects of threats on the stability of soil aggregates. First, a laboratory study explores the effects of elevated temperature and microplastic on fungi-induced aggregation. This work opens the door for the study on effects of two top important global change factors, warming and microplastic pollution, on soil aggregation. Next, a laboratory work investigates the effects of organic matter and microplastic on soil aggregation, revealing the interactive effects of the two factors, which can influence the soil aggregation substantially. Third, a laboratory work was done to explore the interactive effects of microbial diversity and elevated temperature on soil aggregation.

Our investigation of above-mentioned factors on aggregation revealed the interactive effects among these factors. Elevated temperature and microplastic jointly influenced soil aggregation, while the effects varied among fungal species. Then we conducted an experiment to investigate the effects of organic matter and microplastic on aggregation; we assumed that different types of organic matter could induce various levels of microbial activity, which could lead to aggregation to different degrees. We found that effects of microplastic on aggregation depended on the type of added organic matter. We then found that microbial diversity and elevated temperature had interactive effects on aggregation, that microbial community and elevated temperature could influence aggregation by mitigating rates of decomposition.

Our results provide insight about how emerging threats (microplastic, warming and microbial diversity loss) can influence aggregation in the context of different conditions (different species of fungi and organic matter addition). Microplastic fibers affected soil aggregation by interfering with the formation of stable aggregates. Fungi-induced aggregation is sensitive to microplastic and temperature, while those effects will shift in terms of the species of fungi, which play a dominant role in the formation of aggregates. In the presence of fungi that are more able to form the aggregates at elevated temperature, microplastic could lead to more loss in aggregates at elevated temperature. The organic matter-induced aggregation is also vulnerable to microplastic, the magnitude of negative effects of microplastic on aggregate stability depended on the type of added organic matter. Both chapter 2 and chapter 3 indicate that greater soil aggregation activity could lead to increased opportunities for microplastic to be integrated into aggregates, leading to subsequent destabilization of these structures by as

yet unknown mechanisms. These studies contribute to the context dependency of microplastic effects in terrestrial ecosystems. Chapter 4 reveals that elevated temperature decreased the aggregates probably by accelerating decomposition of binding-agents, and also decreased the allocation of soil organic matter into aggregates. Different microbial communities had different reactions to elevate temperature, resulting in interactive effects between elevated temperature and microbial diversity on aggregates and formation of aggregate-protected C.

ZUSAMMENFASSUNG

Bodenaggregate sind Zusammenschlüsse von Bodenpartikeln, die die physikalische Struktur des Bodens bilden. Die Bodenaggregation ist der Schlüssel für zahlreiche Ökosystemfunktionen, einschließlich Bodenbelüftung, Wasserinfiltration und –speicherung und verschiedenster Nährstoffkreislauf.

In dieser Doktorarbeit werden die Auswirkungen verschiedener Faktoren des anthropogenen Umweltwandels auf die Stabilität von Bodenaggregaten untersucht. Zunächst werden in einer Laborstudie die Auswirkungen von erhöhter Temperatur und Mikroplastik auf die pilzinduzierte Aggregation untersucht. Diese Arbeit öffnet die Tür für die Untersuchung der Auswirkungen der beiden wichtigsten Faktoren des globalen Wandels, der Erderwärmung und der Verschmutzung durch Mikroplastik, auf die Bodenaggregation. Als nächstes wurden in einer Laborarbeit die Auswirkungen von pflanzlichem organischem Material und Mikroplastik auf die Bodenaggregation untersucht, wobei die interaktiven Effekte der beiden Faktoren, die die Bodenaggregation wesentlich beeinflussen können, aufgedeckt wurden. Drittens wurde eine Laborarbeit durchgeführt, um die interaktiven Effekte von mikrobieller Diversität und erhöhter Temperatur auf die Bodenaggregation zu untersuchen.

Unsere Untersuchung der oben genannten Faktoren auf die Aggregation zeigten auf, dass es Interaktionen zwischen diesen Faktoren gibt. Erhöhte Temperatur und Mikroplastik beeinflussten gemeinsam die Bodenaggregation, während die Effekte zwischen den Pilzarten variierten. Dann führten wir ein Experiment durch, um die Auswirkungen von organischer Substanz und Mikroplastik auf die Aggregation zu untersuchen; wir nahmen an, dass verschiedene Arten von organischem Material unterschiedliche Niveaus mikrobieller Aktivität induzieren konnten, was zu Aggregation in unterschiedlichem Ausmaß führte. Wir fanden heraus, dass die Auswirkungen von Mikroplastik auf die Aggregation von der Art des hinzugefügten organischen Materials abhängt. Wir fanden außerdem heraus, dass die mikrobielle Diversität und die erhöhte Temperatur interaktive Effekte auf die Aggregation haben, dass also die mikrobielle Gemeinschaft und die erhöhte Temperatur die Aggregation beeinflussen können, indem sie die Zersetzungsraten abschwächen.

Unsere Ergebnisse geben Aufschluss darüber, wie aufkommende Bedrohungen, wie Mikroplastikverschmutzung, Erderwärmung und der Verlust der mikrobiellen Diversität, die Bodenaggregation im Zusammenhang mit verschiedenen Bedingungen (verschiedene Pilzarten und Zugabe von organischem Material) beeinflussen können. Mikroplastikfasern

beeinflussten die Bodenaggregation, indem sie die Bildung von stabilen Aggregaten behinderten. Die pilzinduzierte Aggregation ist empfindlich gegenüber Mikroplastik und Temperatur, wobei sich diese Effekte in Bezug auf die Pilzarten, die eine dominante Rolle bei der Bildung von Aggregaten spielen, verschieben. Bei Vorhandensein von Pilzen, die eher in der Lage sind, die Aggregate bei erhöhter Temperatur zu bilden, kann Mikroplastik zu einem stärkeren Verlust an Aggregaten bei erhöhter Temperatur führen. Die durch organisches, pflanzliches Material induzierte Aggregation ist ebenfalls anfällig für Störungen durch Mikroplastik, wobei das Ausmaß der negativen Auswirkungen von Mikroplastik auf die Aggregatstabilität von der Art des zugesetzten organischen Materials abhängt. Sowohl Kapitel 2 als auch Kapitel 3 deuten darauf hin, dass eine größere Aktivität der Bodenaggregation zu erhöhten Möglichkeiten für Mikroplastik führen könnte, in Aggregate integriert zu werden, was zu einer nachfolgenden Destabilisierung dieser Strukturen durch noch unbekannte Mechanismen führt. Diese Studien tragen zur Kontextabhängigkeit der Auswirkungen von Mikroplastik in terrestrischen Ökosystemen bei. Kapitel 4 zeigt, dass eine erhöhte Temperatur die Aggregate wahrscheinlich durch eine beschleunigte Zersetzung der Bindemittel verringerte und auch die Allokation der organischen Bodensubstanz in die Aggregate verringerte. Verschiedene mikrobielle Gemeinschaften reagierten unterschiedlich auf die erhöhte Temperatur, was zu interaktiven Effekten zwischen erhöhter Temperatur und mikrobieller Diversität auf Aggregate und die Bildung von aggregatgeschütztem C führte.

THESIS OUTLINE

This thesis is a cumulative work, chapter1 is the general introduction, providing background, context and research aims; chapter 2, 3, 4 are three manuscripts with chapter 2, 3 having been published and chapter 4 is prepared for submission to a peer-reviewed journal; chapter 5 is the general discussion, making conceptual linkages between the results of the studies. The references of each manuscript are at the end of the manuscript, the references of the general introduction and the general discussion are provided in the end of the thesis.

Chapter 1: General Introduction

Chapter 2: Liang, Y., Lehmann, A., Ballhausen, M., Muller, L., & Rillig, M. C. (2019). Experimental warming and microplastic fibers jointly influence soil aggregation by saprobic fungi. *Frontiers in Microbiology*, 10, 2018.

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Chapter 3: Liang, Y., Lehmann, A., Yang, G., Leifheit, E. F., & Rillig, M. C. (2021). Effects of microplastic fibers on soil aggregation and enzyme activities are organic matter dependent. *Frontiers in Environmental Science*, 9, 97.

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Chapter 4: Soil carbon protection by aggregates is influenced by temperature and microbial diversity

Author contributions: YL designed, performed the research, and conceived and performed the data analyses. YL, EL, AL and MR wrote the manuscript and contributed to the final version of the manuscript.

Chapter 5: General discussion

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Table 4.1 ANOVA results for the effects of temperature, levels of microbial diversity and the interaction of these factors.

1. GENERAL INTRODUCTION

Soil is an assemblage of primary soil particles, organic matter, water, air and biota living inside. Soil provides many ecosystem functions including gas exchange, water infiltration, erosion resistance, carbon storage (Stewart and Hartge, 1995; Wheeler and von Braun, 2013; Crowther et al., 2016). The physical soil structure is key for soil to fulfil these ecosystem functions.

1.1 Soil physical structure and soil functions

1.1.1 Pore system

Soil pore characteristics are an important indicator of soil quality, determining hydraulic conductivity, aeration, transportation and storage of water and nutrient (Kravchenko et al., 2015). Soil pores are generally categorized into macropores, mesopores and micropores; even though there is no uniform size classification of pores (Tippkötter et al., 2009; Zaffar and Lu, 2015), it is agreed that pores between 0.2 and 30 μm can retain water, pores larger than 30 μm are filled by air (Hardie et al., 2014; Kuncoro et al., 2014; Negassa et al., 2015). Therefore, larger pores are important for water movement which determines hydraulic conductivity (Blackwell et al., 1990; Nachabe, 1995; Kutílek, 2004); micropores are important for water holding capacity, the pore size distribution therefore can determine the water and air distribution in soil. The distribution of water and gas in soil pores influences the survival and activity of microbes, the balanced provision of water and air is crucial for biotic activity (Cook and Orchard, 2008). Moreover, the water obtained in pore system is important for nutrient flow. The diffusive pathway is necessary for substrate diffusion and bacterial motility (Chowdhury et al., 2011), resulting in substrate accessibility to microbes especially bacteria (Poll et al., 2006). However, the soil pores with small size could protect substrate from decomposition, the diameters of bacteria and fungi are typically 0.5 - 1 μm, and 3 - 10 μm, respectively (Killham, 1994). Both fungi and bacteria cannot enter pores smaller than their sizes, such occlusion of organic component may preserve organic carbon for a long time.

The stable soil physical structure fundamentally supports the soil pore system. Aggregate size was found to be correlated with porosity and pore size (Mangalassery et al., 2013). It was assumed that during the binding of microaggregates into macroaggregates, bigger pores would occur, resulting in increased macroaggregates and macropores (Sun and Lu, 2014).

Anthropogenic activities such as tillage decrease aggregate stability, also leading to loss in porosity and larger pores (Wang et al., 2012b; Gao et al., 2019).

1.1.2 Nutrient cycling

Nutrient cycling regulates the transformation and availability of nutrients (Power, 1994), the microbial processes acting on soil organic matter could release soluble components that could be taken up by microbes and plants to support their growth and activity. Soil struture regulates nutrient cycling by influencing water availability, gaseous exchange and nutrient transport. For example, reduced air-filled porosity causes an anaerobic condition, in which denitrification occurs more prevalently; nitrous oxide release would increase during the progress (Linn and Doran, 1984; Dobbie and Smith, 2003; van der Weerden et al., 2012). Soil structure is also related to nutrient leaching, especially nitrate leaching in agricultural fields (Yoo et al., 2014).

1.1.3 C storage

The amount of C stored in soil is approximately 3 times larger than that in the atmosphere, C emission from soil has been considered seriously as a source of greenhouse gases, which accelerates global warming (Oelkers and Cole, 2008). Nearly two-thirds of the total increase in atmospheric CO_2. came from the burning of fossil fuels, the remainder came from terrestrial ecosystems (Lal, 2004).

Physical protection, mineral-association and chemical recalcitrance are key mechanisms for C storage. Recent studies found that the preservation time of C is not relevant to the chemical properties of C , while soil physical structure plays a critical role in controlling microbial processes of soil organic matter (Van Veen and Kuikman, 1990; Dungait et al., 2012). The transformation of nutrients from labile form to stabilized form by microbes contributes to C sequestration in soil substantially (Ma et al., 2018; Liang et al., 2019). The preservation of mineral-associated C lasts for decadal to millennial timescales (Cotrufo et al., 2013). Those microbially derived components, necromass and other small organic components are rapidly associated with minerals and physically incorporated into macroaggregates, thus are protected by aggregate-occlusion additionally. Macroaggregates provide physical protection of particulate organic matter; this slows down the decomposition and promotes the formation of microaggregate within macroaggregates (Six et al., 2004). It was suggested that C

occluded in the microaggregate within macroaggregate was a major mechanism of long-term soil C sequestration in agroecosystems (Kong et al., 2005).

1.1.4 Habitats for microorganisms

Soil structure provides habitats for microbes and other soil animals, contributing to the coexistence of the diverse species living in soil, thus also helping them drive ecosystem functions. It was found that bacteria are predominantly located in pores 3 - 20 µm in diameter, fungi were usually observed in pores >10 µm, the biomass of nematodes was found to depend on the volume of pores 30–90 µm (Hassink et al., 1993). The small pores inside the aggregates could protect bacteria and fungi from predation (Rutherford and Juma, 1992), and also provide partitioned habitats for microorganisms (Chenu et al., 2001; Mummey and Stahl, 2004; Ruamps et al., 2011). The heterogeneous spatial distribution of microorganisms promotes microbial diversity by mitigating competition and predation.

1.1.5 Resistance to erosion

Soil erosion caused by water or wind is one of the main threats to soil, which is accelerated by intensive land use and extreme climate events (Song et al., 2005). It is estimated that the global rate of soil erosion is about 1.4 t $ha^{-1}yr^{-1}$ on all land (Wuepper et al., 2020), the eroded soil per year was predicted as 35.9 Pg (Borrelli et al., 2017). Once the rain, flood or irrigation arrives on the soil surface, if water cannot rapidly filtrate, soil surface run-off will occur, and thus cause soil erosion. (Qiu et al., 2021). The wind velocity also causes erosion, leading to loss in surface dry aggregates, causes air pollution and threats to human health (Dale A. Gillette, 1977; Alfaro et al., 1998).

A stable physical structure is crucial to resist erosion (Eldridge and Leys, 2003; Tisdall et al., 2012), the size and stability of the soil particles is reversely related to the soil erodibility (Hevia et al., 2007; Zamani and Mahmoodabadi, 2013; Zobeck et al., 2013).

1.2 Factors influencing the formation and stability of aggregates

Soil aggregates are the configuration and assemblage of soil particles, aggregates and associated pore networks (Lal, 1991), with the stability of aggregates a key indicator of soil physical structure. According to the aggregate hierarchy model, primary soil particles are assembled into microaggregates, then microaggregates cluster into macroaggregates;

microaggregates can also form within the macroaggregates (Tisdall and Oades, 1982; Oades and Waters, 1991; Six et al., 2000).

1.2.1 Physical interactions

Wet-dry cycling can cause uneven wetting and drying in soil, subsequently causing fractures and cracks between soil particles, thus decreasing the size of aggregates (Hadas, 1990; Ma et al., 2015). Nevertheless, wetting-caused swelling can increase contact between organic and mineral compounds that favours bond formation in some cases; such contrary wet-drying cycle effects on aggregation were attributed to the differences in clay types, cycle numbers, water content, and other factors (Singer et al., 1992; Ma et al., 2015).

Freeze-thaw cycles generally reduce stable aggregates, due to the ice crystalization leading to the breakdown of aggregates; however, the effects are dependent on the soil water content, number of cycles and soil type (Benoit, 1973; Oztas and Fayetorbay, 2003; Wang et al., 2012a). It was found that freeze-thaw cycles decreased aggregates more pronouncely with high moisture contents, but could exhibit a positive effects on aggregate stability with an intermediate water content (Li and Fan, 2014).

1.2.2 Chemical interactions

The association among clay, polyvalent cations and organic matter is responsible for the stability of soil aggregates, the effects depending on the clay minerology (Oades, 1984). Clays are mostly aluminosilicates and a range of oxides, hydroxides and oxyhydroxides, the main types of clay aluminosilicates are kaolinite, illite and smectite (Churchman et al., 1993). Unlike smectites, kaolinites and illites do not swell when wetted (Dixon, 1991; Pal et al., 2009), and they have lower cation exchange capacity and specific surface area than smectites (Dixon, 1991; Churchman et al., 2006). The swelling may cause aggregate slaking (Bartoli et al., 1988), therefore soils with high content of kaolinite are more stable than those with high content of smectite (Amézketa, 1999).

Fe, Al, and Mn oxides can bridge between negatively charged organic carbon and negatively charged clays, and simultaneously stabilize aggregates (Muneer and Oades, 1989); such binding mechanisms are especially important for the stabilization of microaggregates (Oades, 1984).

1.2.3 Biochemical and biological factors

Geophagous animals such as earthworms contribute to aggregation greatly (Bossuyt et al., 2006; Fonte and Six, 2010). The earthworm cast, a mixture of soil and organic matter, can promote the formation of stable aggregates (Bossuyt et al., 2005). Earthworm burrows could enhance the water filtration, gas exchange and root growth, thus also promote aggregation (Lee and Foster, 1991; Schon et al., 2017).

Fungi were found to have the most substantial positive effects on aggregate stability among biotic factors (Lehmann et al., 2017). The fungal hyphal network could entangle soil particles and organic matter, the fungi produced polymers act as binding agents, altogether contributing to the formation and stabilization of aggregates (Chenu, 1989; Degens, 1997b). The fungal capacity to stabilize aggregates varies among species (Lehmann et al., 2020). Mycorrhizal fungi release proteins that are potentially important for stable soil structure formation (Rillig and Mummey, 2006). Bacteria can also contribute to aggregation by secreting mucilage, polysaccharides and other extracellular compounds that act as gluing agents (Tisdall, 1994).

Plant can increase aggregates by root enmeshment (Totsche et al., 2018), plant derived polysaccharides and humic substances also directly contribute to aggregation (Mbagwu and Piccolo, 1989). Moreover, root exudates and plant litter material can stimulate microbial activity and thus promote the production of microbially derived components, acting as the binding agents (Angers and Caron, 1998).

1.3 Threats to soil aggregates

Our soil is facing many unprecedented challenges. Anthropogenic activity influences the global climate, and also brings new threats to terrestrial ecosystem, such as warming, microplastic pollution and microbial diversity loss in soil (Butchart et al., 2010; Rillig, 2012; Allen et al., 2014a). These changes act upon the soil system with far-reaching but poorly understood consequences on soil physical structure.

1.3.1 Warming

Human activity increased global temperature compared to pre-industrial times (1850-1900) and will likely induce 1.5 °C warmer temperatures around 2040 (Shukla et al., 2019). Warming is considered as an urgent and potentially irreversible threat to natural ecosystem. Under global

warming, the elevated temperature could simulate microbial activity (Pietikäinen et al., 2005), thus accelerating organic matter decomposition, the simultaneously boosted respiration (Zogg et al., 1997; Allison and Treseder, 2008; Dang et al., 2009; Bell et al., 2010) can cause positive feedbacks to warming. The stimulated respiration might be attributed to several reasons. First, higher temperature might accelerate the substrate diffusion, thus the possibility of substrate bound to enzyme's active site increases (Monson et al., 2006). Second, the higher temperature causes the desorption of organic C from the SOM complex (Kaiser et al., 2001). Third, higher temperature could select for fungi capable of degrading recalcitrant C (Treseder et al., 2016). Therefore, more substrates are available to microbial decomposers due to warming, the decomposition rate increases as a result.

Nevertheless, data on the influence of soil warming on soil aggregation are very limited. We assume that soil aggregation is very likely to be influenced by warming. Higher temperature increases the growth and activity of microbes, accelerates decomposition, leads to greater hyphae network and microbially-derived components, which benefit stable aggregate formation. However, we assume that the soil C depletion might cause the degradation of binding agents of stable aggregates. It was found that warming substantially decreased the microbial C fraction (Liang and Balser, 2012); higher temperature could induce shifts in the microbial community, which had more microbes with lower biomass (Li et al., 2019); the higher temperature could also change the microbial physiology, leading to lower carbon use efficiency. Therefore, higher temperature could decrease the microbial contribution to stable C and likely decrease the binding agents, leading to less stable aggregates.

1.3.2 Microplastic

Microplastic has been proposed as a new global change factor (Rillig and Lehmann, 2020), possessing pervasive and persistent influences on ecosystem. Microplastic is widely studied in marine ecosystems (Eriksen et al., 2014; Bergmann et al., 2015; Jambeck et al., 2015), and only in recent years has attention shifted to terrestrial ecosystems (Rillig, 2012; Bläsing and Amelung, 2018; de Souza Machado et al., 2018a; Zhou et al., 2020b). Microplastics are defined as plastics ranging in size from 5 mm to 1 µm (Moore, 2008; Barnes et al., 2009; Zhou et al., 2018). Due to the different uses and purpose of plastic, plastics are produced in different polymer types (e.g., polyester, polyethylene, polyacrylic, polypropylene) (Geyer et al., 2017), are modified by different additives (Bandow et al., 2017), appear in different shapes (e.g.,

fragment, film, fiber) (Rillig et al., 2019), and thus microplastics have various inherent properties.
Microplastic fibers are a dominant shape encountered in microplastic pollution. Microplastic fibers derived from textiles are commonly discovered in wastewater (Pirc et al., 2016; Athey et al., 2020), sediments (Browne et al., 2011) and air deposition (Cai et al., 2017). It was found that particle number of microplastics released from the treated effluent to a recipient river could be 2.2×10^7 particles per day on average (Blair et al., 2019); the microplastics particles accumulated in sediments had an average concentration of 34 ± 10 items/kg sediments (Zobkov and Esiukova, 2017); hundreds of particles are deposited from the atmosphere per square meter per day (Dris et al., 2017; Brahney et al., 2020). Microplastic fibers derived from textiles are likely to be present in sludge and biosolids in the end, which would be applied on agricultural fields as fertilizer (Henry et al., 2019; Crossman et al., 2020), resulting in a considerable pollution of microplastic fibers in soils (van den Berg et al., 2020; Zhou et al., 2020a). Anthropogenic activities, such as tillage, and movement of soil animals can accelerate the incorporation of microplastic fibers into the soil (Huerta Lwanga et al., 2017; Rillig et al., 2017). Microplastic fibers have been shown to influence soil quality and health by being detrimental to soil aggregate stability (de Souza Machado et al., 2018b; Lehmann et al., 2019a) and altering microbial activity (Liu et al., 2017; Huang et al., 2019). Microplastic could also change the microbial community, as microplastic could be a potential substrate and habitat to microbes (Zhang et al., 2019). The shift in microbial community and microbial activity could lead to changes in microbial functions, the decrease in stability of aggregates can be one of the consequences. However, soil aggregation is determined by many factors, the effects of microplastic fiber on soil aggregation under different soil conditions are not well studied.

1.3.3 Loss of microbial diversity

Our earth is facing biodiversity loss due to pressures such as intensive land use, pollution, climate change, alien species invasion, resource consumption (Butchart et al., 2010; Zhang et al., 2016); biodiversity continues to decrease despite of some conservation successes (Rands et al., 2010). The loss in biodiversity could alter functions of ecosystems and their services (Cardinale et al., 2012), such as productivity of ecosystems or climate regulation.

In terrestrial ecosystems, microbial diversity is pivotal to maintaining ecosystem multifunctionality (Delgado-Baquerizo et al., 2016). For example, microbial biodiversity is associated with the microbial processes acting on soil organic matter (Tardy et al., 2015); for

example, microbial diversity can promote decomposition (Maron et al., 2018), thus supporting nutrient cycling, soil fertility and crop productivity. It is generally assumed that lignin degradation can be altered by microbial diversity, because processing of such plant physical structural polymers can only be carried out by a few organisms (Cox et al., 2001). It was also found that degradation of plant sugar decreased due to microbial diversity loss (Baumann et al., 2013). However, some studies found the loss in microbial diversity increased soil respiration (Zhang and Zhang, 2016).

Under global warming, we assume the loss in diversity could cause unprecedented influence on terrestrial ecosystem. Many studies found that microbial diversity increases the stability of ecosystems functions under stress (Pennekamp et al., 2018; Yang et al., 2021), decreased soil microbial diversity could lead to vulnerable ecosystem that fail to resist warming (Yang et al., 2020). However, the interactive effects of microbial diversity and warming is unknown.

1.4 Research Aims

The aim of this doctoral work is to target the gaps identified above. Chapter 2 reports on an experiment investigating the effects of experimental warming and microplastic on fungi-induced aggregation; Chapter 3 reports on the interactive effects of microplastic fibers and organic matter on soil aggregation. Chapter 4 reports on an experiment exploring the interactive effects of experimental warming and microbial diversity on soil aggregation; These three works provide insight about influence of warming or microplastic or microbial diversity loss on soil aggregation in certain contexts that reflect relatively realistic scenarios.

2. ELEVATED TEMPERATURE AND MICROPLATIC FIBERS JOINTLY INFLUENCE SOIL AGGREGATION BY SAPROBIC FUNGI

Yun Liang, Anika Lehmann, Max-Bernhard Ballhausen, Ludo Muller and Matthias C. Rillig

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Keywords: abrupt, gradual, temperature, microplastic, fungi, soil aggregation.

2.1 Abstract

Microplastic pollution and increasing temperature have potential to influence soil quality; yet little is known about their effects on soil aggregation, a key determinant of soil quality. Given the importance of fungi for soil aggregation, we investigated the impacts of increasing temperature and microplastic fibers on aggregation by carrying out a soil incubation experiment in which we inoculated soil individually with 5 specific strains of soil saprobic fungi. Our treatments were temperature (ambient temperature of 25°C or temperature increased by 3°C, abruptly versus gradually) and microplastic fibers (control and 0.4% w/ w). We evaluated the percentage of water stable aggregates (WSA) and hydrolysis of fluorescein diacetate (FDA) as an indicator of fungal biomass. Microplastic fiber addition was the main factor influencing the WSA, decreasing the percentage of WSA except in soil incubated with strain RLCS 01, and mitigated the effects of temperature or even caused more pronounced decrease in WSA under increasing temperature. We also observed clear differences between temperature change patterns. Our study shows that the interactive effects of warming and microplastic fibers are important to consider when evaluating effects of global change on soil aggregation and potentially other soil processes.

2.2 Introduction

Our soils are confronted with an unprecedented change: due to anthropogenic influence the climate is globally changing but also new threats of contamination are emerging (Allen et al., 2014b; de Souza Machado et al., 2018a). These changes act upon the soil system with far-reaching but poorly understood consequences on soil biota, soil functions (e.g. gas exchange, water infiltration, erosion resistance (Stewart and Hartge, 1995)) and ecosystem services (e.g. carbon storage, food production (Wheeler and von Braun, 2013; Crowther et al., 2016)). A key aspect of soil to consider is its structure: the configuration and assemblage of soil particles, aggregates and associated pore networks (Lal, 1991). Soil structure can be affected by aspects of global change and in turn controls key aspects of soil biota and soil functions; thus, in order to mechanistically understand effects of global change on soil we need to assess consequences for soil structure and the process leading to soil structure, soil aggregation. The process of soil aggregation encompasses the process components formation, stabilization and

disintegration; all working simultaneously under natural conditions giving rise to soil aggregates which represent building blocks of soil structure.

Soil aggregation is a biota-driven process with soil microbes in general and filamentous soil fungi in particular as substantial contributors (Forster, 1990; Degens, 1997a; Lehmann and Rillig, 2015; Lehmann et al., 2017). Soil fungi contribute to and are affected by their complex and heterogeneous environment in multiple ways: during their foraging activities, hyphae of soil fungi entangle and enmesh soil particles (Tisdall, 1991; Daynes et al., 2012a; Gupta and Germida, 2015) and aggregates, while also exuding exo-biopolymers functioning as binding agents (Chenu, 1989; Caesar-Tonthat, 2002; Daynes et al., 2012b). Conversely, fungal growth and activity itself is modulated by the biotic, abiotic and spatial context of the soil matrix (Harris et al., 2003; Boswell et al., 2006).

It is widely acknowledged that temperature is a crucial factor determining activity of soil microbes (Pietikäinen et al., 2005), with higher temperature stimulating biological activities, such as respiration, growth rate, decomposition, extracellular enzyme activity, and secretion of metabolites (Zogg et al., 1997; Allison and Treseder, 2008; Dang et al., 2009; Bell et al., 2010); this also holds true for soil fungi (Jackson et al., 1991; Loera et al., 2011; A'Bear et al., 2012). Thereby, elevated temperature potentially influences soil processes driven by microbes, including soil aggregation. Nevertheless, data on the influence of soil warming on soil fungal contributions to soil aggregation are very limited. The limited data available suggest that with increasing soil temperature soil aggregate stability decreases (Rillig et al., 2002; Guan et al., 2018). The role of soil fungi in this decrease of soil aggregation with increasing temperature is not clear.

Studies on increasing temperature commonly increase temperature abruptly, which ignores the fact that temperature might also rise gradually in nature. The limited suite of studies applying such a gradual approach detected less pronounced effects in plant and soil microbe activity than under abrupt changes (for CO_2 and salinity (Klironomos et al., 2005a; Yan and Marschner, 2013). For soil microbes (e.g., arbuscular mycorrhizal fungi), shifts in community composition and functionality were detectable (Klironomos et al., 2005b), suggesting that slower, more gradual rates of environmental change can result in more adapted and thus more resilient, final populations of organisms or fewer extinctions. In addition, a simulation study demonstrated specifically for soil warming that an abrupt change resulted in larger soil respiration than did gradual change (Shen et al., 2009). Considering these data, it is necessary

to assess the effects of abrupt vs. gradual change when investigating the impact of increasing temperature on fungal contributions to soil aggregation, because such a comparison may yield more robust insights.

Soils are exposed to a multitude of global change factors some of which recently moved into research focus; among these is microplastics - a group of pervasive, ubiquitous, anthropogenic contaminants (de Souza Machado et al., 2018a). Microplastics comprise chemically diverse polymers of varying shapes and structures, with a size range from 5 mm to 1 µm which are produced as (primary) or fragmented into (secondary) micro-sized plastic particles via environmental factors (Hartmann et al., 2019). Microplastics can be found world-wide not only in marine but also terrestrial ecosystems, which received considerable attention recently (Rillig, 2012; Huerta Lwanga et al., 2016; Bläsing and Amelung, 2018). The limited data available so far suggests that microplastic particles, especially fibers, have indirect effects on soil aggregation via the soil microbial pathway by physically changing soil properties (e.g. decreasing soil bulk density (de Souza Machado et al., 2018c)). By changing the conditions in the soil matrix, a shift in fungal growth and activity can be expected. There are so far no studies evaluating the effect of microplastics on soil fungi; a significant gap in our knowledge, which has to be approached to understand microplastic impact on soil aggregation. We assume that lower bulk density, particularly due to microplastic fiber addition, leads to higher aeration-dependent microbial activities, moreover, the increased pore space likely provides favorable conditions for hyphal extension (Elliott et al., 1988; Wang et al., 2017).

Although environmental factors affect soils in combination, studies tend to test these factors in isolation. This hampers our understanding of potential interactive effects on the targeted study systems. To contribute to the identified research gaps in effects of environmental factors on fungal mediated soil aggregation, we conducted a laboratory study with soil inoculated with filamentous soil fungal strains and microplastic fiber and temperature (with both abrupt and gradual increase) treatments. We aimed to investigate interactive effects of two global change drivers of significant importance for soil and fungal systems. We test the following hypotheses: 1. Rising temperature will lead to higher fungal activity resulting in higher fungal contribution to soil aggregation as compared to control settings. 2. Gradually rising temperatures will affect fungal activity and soil aggregation less than abrupt temperature change. 3. Microplastic fibers will promote fungal activity and fungal

contributions to soil aggregation. 4. Microplastic fibers and increasing temperature will interact in their effects on fungal activity and soil aggregation.

2.3 Materials and methods

2.3.1 Microfiber

In this experiment, we focus on microplastic fibers, since in a previous experiment plastic fibers elicited stronger effects on soil aggregation than fragments or beads (de Souza Machado et al., 2018c). Additionally, recent studies found that atmospheric deposition of microplastic fibers is an important source of soil contamination (Dris et al., 2016; Zhang and Liu, 2018; Henry et al., 2019). From the many available polymer types, we chose polyacrylic (PAN) fibres, which are produced from acrylonitrile. Polyacrylic fibers are easy to process and can affect soil aggregation (de Souza Machado et al., 2018c). We produced microfibers by manually cutting 100% acrylic "Bravo" yarn (schachenmayr.com) into length ranges from 0.37 to 3.14 mm (Appendix Figure A2.1). The diameter of these polyacrylic fibers was 0.026± 0.005 mm. The microfibers were sterilized by microwaving and subsequently added to the soil as 0.4% (w/w), 0.4% was determined as the upper limit concentration in previous study in our lab, which was determined based on the highest concentration at which soils experienced minor changes in volume after the addition of linear microplastics (de Souza Machado et al., 2018c). The sterilized fibers were placed on PDA (see below) plates, no contamination was observed after 7 days.

2.3.2 Fungi inoculum preparation

We selected five fungal strains from a set of filamentous fungi maintained in our lab (Andrade-Linares et al., 2016), the method of identification were in Appendix Table A2.1, originally isolated from a natural semi-arid grassland (Mallnow Lebus, Brandenburg, Germany, 52°27.7780' N, 14°29.3490' E): RLCS 01, RLCS 05, RLCS 06, RLCS 07, RLCS 08, the species are: *Mucor fragilis, Fusarium sp., Chaetomium angustispirale, Amphisphaeriaceae strain 1, Gibberella tricincta*, respectively (Appendix Table A2.1). Instead of referring to a species name, we address our strains solely with the identifier RLCS following with the strain-specific number. Strains are sorted by colony radial extension rate from high to low (Appendix Table A2.1). The fungal strain information table is in the Appendix Table A2.1. The fungal strains were filamentous, saprobic fungi, selected for comparable growth rate on potato dextrose agar (PDA X931.2, Roth, Germany) (Zheng et al.,

2018), and optimum growth temperature around 25 °C (data not shown here). They exerted different ability in forming soil aggregates (Lehmann et al., 2019b), RLCS 01 is the poorest soil aggregator while RLCS 08 is the best. Though fungi may not performance the same each time, by this, we avoid confounding effects by differences in produced fungal biomass, while covering variance of soil aggregation capability. Fungi were cultured in potato dextrose broth (PDB CP74.2, Roth, Germany) for 4 days on a rotary shaker (New Brunswick™, Eppendorf) at 150 rpm. Hyphal fragments for inoculation were produced by disrupting the mycelium with glass beads (diameter = 0.25- 0.5 mm; Roth, Germany) shaken on a vortex mixer at highest speed for 1 min (Vortex-Genie, Scientific Industries, U.S.A). The resulting suspension was passed through a 20 µm nylon membrane to retrieve fungal fragments of a homogeneous size. In order to minimize the difference in propagule numbers, fungal mycelium fragments were diluted in PDB to a concentration of 40-100 fragments per 20 µl. In a preliminary test, we plated the mycelium fragment solutions of the different strains and counted the emerging colonies (Appendix Table A2.2). For each strain, we prepared final inoculum suspensions with the appropriate dilution factor. We used 20 µl of mycelium fragment-PDB suspension for inoculation and 20 µL of mycelium-free PDB for the controls.

2.3.3 Incubated soil

Fresh soil was collected from Mallnow Lebus, a dry grassland in a natural reserve (Brandenburg, Germany, 52°27.7780' N, 14°29.3490' E) characterized as a sandy loam soil texture(Horn et al., 2015), from which the focal fungal strains had been originally isolated In this study, soil was sieved (1 mm) and thoroughly homogenized; this method is commonly used to measure macroaggregate formation in laboratory incubations (De Gryze et al., 2006). We placed 10 g (± 0.01 g) of the soil in test tubes which were autoclaved twice (121°C for 20min). After drying the soil at 60°C, we transferred the sterilized soil into Petri dishes (60 × 15 mm) to mix the sterilized soil with 40 mg of microfiber and 20 µl of fungal homogenate, depending on the treatment. The soil mixture was uniformly wetted with sterilized distilled water amended with glucose to keep water content at 80% water holding capacity and to ensure that every microcosm received 1.89 mg C-glucose to stimulate fungal growth. Glucose as an easily decomposable substrate that can rapidly stimulate the growth of soil microorganisms and has no direct effect on macroaggregation (Abiven et al., 2008), and our own preliminary data (not shown here) also confirmed this. Controls without inoculation also received the same amount of water and glucose.

All samples were sealed with parafilm and placed into plastic boxes with covers. We placed wet paper towels inside the boxes to maintain high air humidity to prevent the soil from drying. Samples were incubated for 42 days during which the temperature treatments were applied.

2.3.4 Temperature treatment

We incubated all microcosms at ambient temperature (25 °C) for the first 7 days to let inoculants establish. To realize the gradually rising temperature, we increased temperature by 3 °C from 25 °C to 28 °C at the speed of 0.15 °C day^{-1} from day 8 to day 27. For the abrupt temperature treatment, the 3 °C increase was applied on day 18. This difference in timing ensured that the mean temperature was the same for the gradual and abrupt temperature treatment (Appendix Figure A2.2). We increased temperature by 3°C based on climate models, which predict that the mean annual global surface temperature will increase by 1-3.5 °C until 2100 (Beckage et al., 2018).

2.3.5 Experimental design

Each of the five fungal isolates and the control were exposed to the combinations of microfiber (yes (M)/no (C)) and temperature (ambient (0), abrupt (+3abrupt) and gradual (+3gradual)), resulting in 6 treatments: T_0-C, T_0-M, $T_{+3abrupt}$-C, $T_{+3abrupt}$-M, $T_{+3gradual}$-C, $T_{+3gradual}$-M. Each treatment had 7 replicates, for a total of 252 experimental units. The units under elevated temperature were placed in three independent incubators split in groups of three and two-times two units. By this approach, we were able to also replicate increased temperature and account for the variability among incubators.

2.3.6 Aggregate stability

We dried soil samples at 40 °C and then sieved them (2 mm). Before wet-sieving, 4.0 g dry soil was placed into sieves for capillary rewetting and subsequently submerged in deionized water for 5 min. We used 0.25 mm sieves to test the stability of the soil fraction >0.25 mm (macroaggregate) against water as disintegrating force. For the test, sieves carrying the wetted soil samples were placed in a wet-sieving machine (Eijkelkamp, Netherlands) for 3 min. The fractions left on the sieves were dried at 60 °C for 24 h. The coarse matter (sand and organic matter fraction) was extracted before calculation of the percent water stable aggregates (WSA):

$$\%\boldsymbol{WSA} = \frac{\textbf{water stable fraction} - \textbf{coarse matter}}{\boldsymbol{4g - coarse\ matter}}$$

2.3.7 Hydrolysis of fluorescein diacetate

We measured he fluorescein diacetate (FDA) hydrolytic activity to indicate fungal activity, which is considered as an indicator of fungal biomass (Gaspar et al., 2001). We quantified the hydrolysis of fluorescein diacetate (FDA, Sigma-Aldrich) by adding 0.75 ml of 100 mM of potassium phosphate buffer (pH 7.6) and 0.1 ml of 2 mg/ml FDA (Adam and Duncan, 2001) to 0.5 g of dry soil. The reaction mixture was placed on a shaker (New Brunswick™, Eppendorf) at 150 rpm at 30 °C for 2 h. The reaction was terminated by adding 0.75 ml of acetone (Roth, Germany). After shaking and centrifugation of samples for 5 min at 3000 rpm, the extracted fluorescein was determined at 490 nm by spectrophotometry (UV-3100 PC, VWRTM, Germany).

2.3.8 Statistical analysis

We analyzed the effects of elevated temperature, microfiber addition and fungal species by using three-way ANOVAs. We used Shapiro Wilk test and Bartlett test to check the normality of residuals and the homogeneity of variances, respectively, with a p-value cutoff of 0.05. We compared the difference between the treatments according to Duncan's test or Student's t-test at a probability level of 5%. All statistics were conducted in R (R Core Team, 2017) with the basic packages, while the plots were created with the graphic package 'ggplot2' (Hadley and Winston, 2016).

2.4 Results

2.4.1 Fungal contributions to soil aggregation

We investigated under ambient, non-contaminated conditions how fungal inoculation affected soil aggregation. We detected a significantly higher ($p < 0.05$) macroaggregate stability than in control samples with the exception of the strain RLCS 01 (Figure 2.1 and Appendix Table A2.3), the strain RLCS 06 had the strongest positive effect (Appendix Table A2.3) leading to a 227.49% increase in the percentage of WSA. The ability to form stable aggregates at 25 °C varies among fungi.

Table 2.1 ANOVA results for the effects of fungi, temperature, plastic micofibers and the interaction of these factors on percentage of water-stable macroaggregates.

Source	**F value**	**P value**
Fungi	96.489	< 0.0001***
Temperature	4.066	0.018*
Microfibers	68.136	< 0.0001***
Fungi × Temperature	3.322	0.0005***
Fungi × Microfibers	38.384	< 0.0001***
Temperature × Microfibers	2.351	0.098
Fungi × Temperature × Microfibers	3.438	0.0003***

The asterisks, *, **, *** denote significant differences (p < 0.05, < 0.01 and < 0.001, respectively)

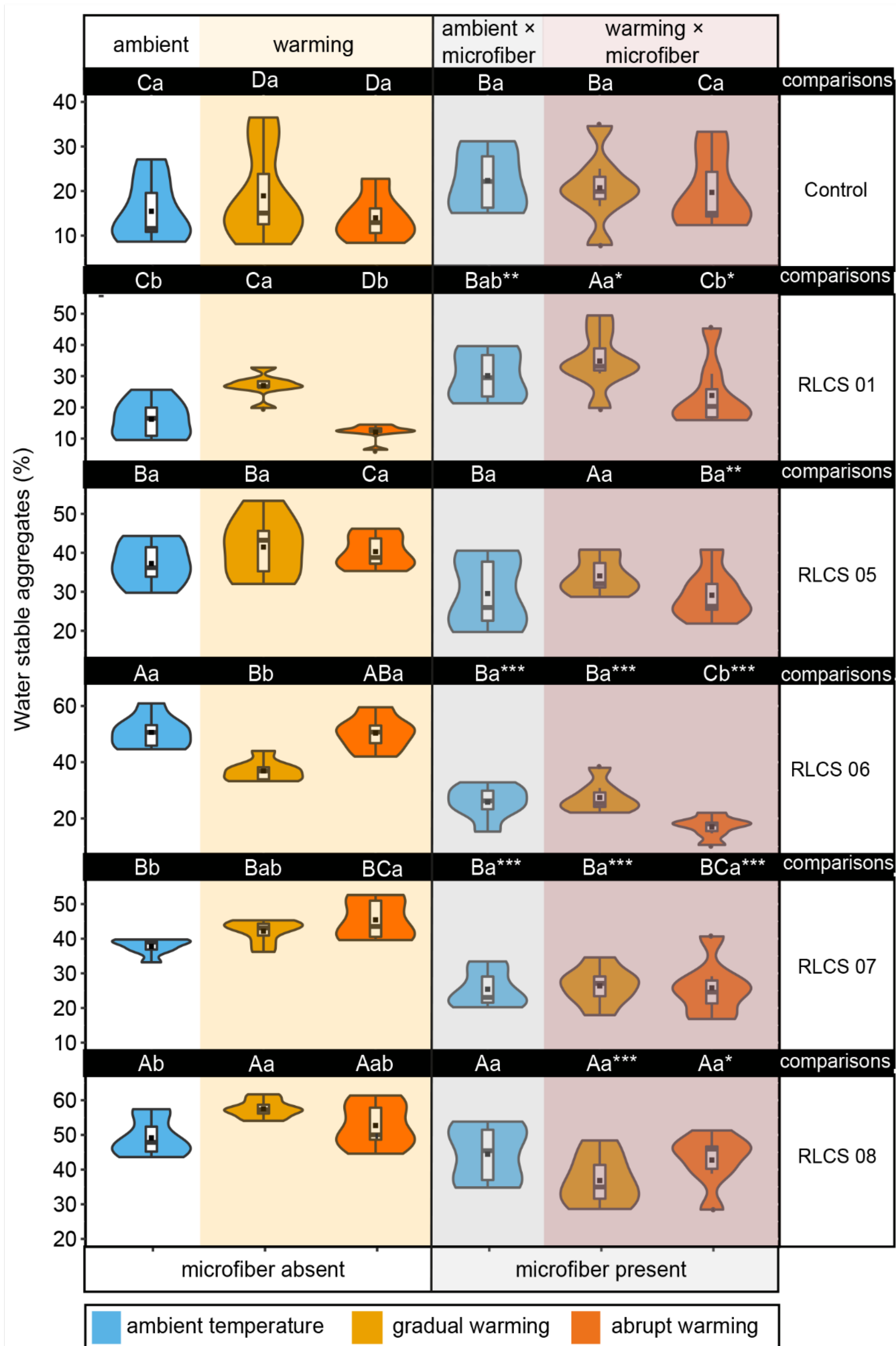

Figure 2.1 The effects of temperature and microplastic fiber addition on the percentage of water stable aggregates depicted as violin/ box plots. Different capital letters indicate significant differences (Duncan's test, $p < 0.05$) among different fungi for the same treatment (i.e. in columns), and lowercase letters indicate significant differences (Duncan's test, p <

0.05) among temperature treatments in terms of microplastic being present or absent (that is, referring to each part of a row). The asterisks, *, **, *** denote significant differences (t-test, $p < 0.05$, < 0.01 and < 0.001, respectively) between microplastic being present and absent within the same temperature treatment. The dots (•) represent outliers, squares (▪) represent means.

2.4.2 Temperature effects on fungal contributions to soil aggregation

Elevated temperature alone caused increase in WSA for soil with RLCS 01, RLCS 07 and RLCS 08, caused no change in WSA for soil with RLCS 05. Nevertheless, WSA of soil with RLCS 06 significantly decreased ($p < 0.05$) under gradually elevated temperature.

In case of soil with RLCS 01 and RLCS 06, we observed a significant difference ($p < 0.05$) in the percentage of WSA between abrupt change and gradual change.

2.4.3 Plastic microfiber effects on fungal contribution to soil aggregation

Regardless of rising temperature, microfiber addition dramatically decreased the percentage of WSA in soil with RLCS 06 and RLCS 07 by 49.08% and 32.69%, respectively, but significantly increased the percentage of WSA in soil with RLCS 01 by 86.33%. Microfiber addition alone, in the absence of fungi, had no significant effect ($p > 0.05$) on the percentage of WSA.

2.4.4 Interactive effects of temperature and plastic microfibers on fungal contributions to soil aggregation

We observed significant interactive effects between elevated temperature and plastic microfibers (Table 2.1). For soil with RLCS 01, RLCS 07 and RLCS 08, rising temperature caused a significant increase ($p < 0.05$) in the percentage of WSA for treatment $T_{+3abrupt}$-C, $T_{+3gradual}$-C, $T_{+3gradual}$-C, compared to control (T_0-C). Such increases disappeared in the presence of microfibers, resulting in no significant difference in WSA between ambient and elevated temperature.

The effects of rising temperature on WSA might even shift in the presence of microfibers. For soil with RLCS 07 and RLCS 08, abruptly and gradually elevated temperature, which respectively caused the highest stability, unexpectedly led to the lowest WSA in the presence

of microfibers, indicating that effects of rising temperature might even turn to negative, leading to greater loss in the percentage of WSA than microfiber alone. Moreover, for soil with RLCS 06, though gradually elevated temperature had negative effect, no significant difference ($p < 0.05$) was observed in the percentage of WSA between $T_{+3agradual}$-M and T_0-M in the presence of microfibers.

In our study, the effect of microfibers also depended on temperature change patterns. Under abruptly elevated temperature, for soil with RLCS 06, microfibers caused substantial decreases in the percentage of WSA by 66.3%, which were higher than the decreases of 25.68% under gradually elevated temperature. Under abruptly elevated temperature, for soil with RLCS 08, microfibers caused a greater decrease in the percentage of WSA than under abruptly increased temperature.

Table 2.2 ANOVA results for the effects of fungi, temperature (abrupt and gradually rising temperatures), plastic microfiber and the interaction of these factors on FDA hydrolysis activity of soil.

Source	**F value**	**Pr (>F)**
Fungi	22.58	< 0.0001***
Temperature	3.12	0.047*
Microfibers	2.40	0.12
Fungi × Temperature	1.44	0.18
Fungi × Microfibers	18.94	< 0.0001***
Temperature × Microfibers	0.34	0.71
Fungi × Temperature × Microfibers	7.21	< 0.0001***

The asterisks, *, **, *** denote significant differences (p < 0.05, < 0.01 and < 0.001, respectively)

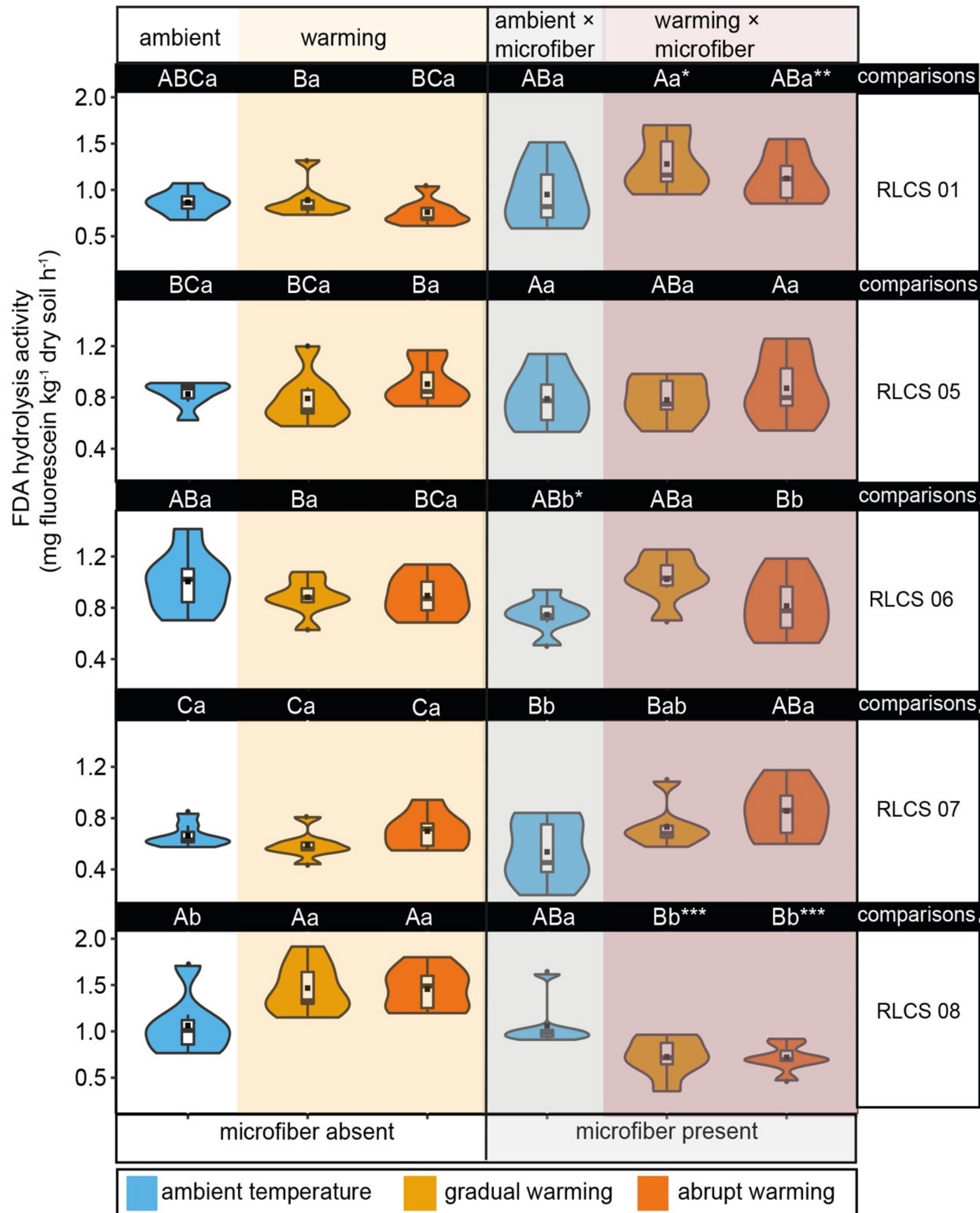

Figure 2.2 The effects of temperature and microplastic fiber addition on FDA hydrolysis activity depicted as violin/ box plots. Different capital letters indicate significant differences (Duncan's test, $p < 0.05$) among different fungi for the same treatment (i.e. in columns), and lowercase letters indicate significant differences (Duncan's test, $p < 0.05$) among temperature treatments in terms of microplastic being present or absent (that is, referring to each part of a row). The asterisks, *, **, *** denote significant differences (t-test, $p < 0.05$, < 0.01 and < 0.001, respectively) between microplastic being present and absent within the same temperature treatment. The dots (•) represent outliers, squares (▪) represent means.

2.4.5 FDA hydrolysis activity

The interactive effect of increasing temperature and microfibers on FDA hydrolysis activity was significant (Table 2.2). Generally, FDA hydrolysis activity was positively correlated with WSA, especially for soil with RLCS 01 (r = 0.44, p = 0.0039) and RLCS 08 (r = 0.61, p = 2.1e-05). Rising temperature alone caused significant increase ($p < 0.05$) in FDA hydrolysis activity for soil with RLCS 01, RLCS 07 and RLCS 08, which is consistent with the WSA data. Microfiber alone led to higher FDA in soil with RLCS 01 while causing lower FDA in soil with RLCS 06 and RLCS 08 (Figure 2.2).

2.5 Discussion

We here present data from a laboratory study investigating for the first time the impact of plastic microfibers and temperature increase on fungal effects on soil aggregation. We find that effects were dependent on the fungal species, and that microfibers and temperature interacted in complex ways. In the following, we discuss results in a progression from main effects to their interaction.

We found the ability to form stable aggregates at ambient temperature varies among fungi. Previous work demonstrated that traits of fungi have different roles in formation, stabilization and disintegration of aggregation (Lehmann and Rillig, 2015), and thus fungi that vary in traits are expected to differently affect WSA. Basically, fungal strains of the Ascomycota could form aggregates efficiently while strains belonged to the Mucoromycota are poor aggregate formers (Lynch and Elliott, 1983; Tisdall et al., 2012). According to our previous study, the traits of fungi contribute to the aggregate formation include high biomass density, large hyphal diameters, low leucine aminopeptidase activity, et al. (Lehmann et al., 2019b). The increase in WSA due to increasing temperature might be attributed to enhanced hyphal growth and higher levels of secretion of bindings agents; however, warming also accelerates decomposition of such binding agents, which are important for forming stable soil aggregates (Tisdall and Oades, 1982), resulting in breakdown of aggregates. Given these different mechanisms, the overall effects might be species-specific. RLCS 06 (*Chaetomium angustispirale*) as a cellulolytic fungus has a strong ability to degrade cellulose(Sahab Yadav and Bagool, 2015), and thus it is possible that RLCS 06 accelerated decomposition of soil organic matter under gradually increased temperature, resulting in less WSA.

Even though we hypothesized that gradually increased temperature would lead to milder effects on WSA, changes in the percentage of WSA caused by gradually rising temperature

were still substantial in some cases, which probably is due to the rate of change being relatively high (0.15 °C day^{-1}), thus potentially not permitting fungi to adjust to the warming. However, the significant difference ($p < 0.05$) in the percentage of WSA between temperature change patterns, for soil with RLCS 01 and RLCS 06 , still underscored that gradually rising temperature should be explored more in laboratory warming experiments to assess differential effects.

Decreases in WSA due to microfiber addition were observed in previous studies (de Souza Machado et al., 2018c; Zhang and Liu, 2018). Polyacrylic fibers significantly decreased the amount of water stable aggregates (de Souza Machado et al., 2018c). Possible reasons include plastic fibers preventing microaggregates from effectively being integrated into macroaggregates (Zhang and Liu, 2018), and the inclusion of microfibers within macroaggregates (Bläsing and Amelung, 2018), finally leading to less stable macroaggregates. However, RLCS 01 led to higher WSA when microfibers were added, a response for which an explanation is not clear. Microfiber increased FDA in soil with RLCS 01, therefore this strain was likely able to maintain or even have higher activity in the presence of microfiber, which may partially explain the observed effect. Previous studies found some fungal species could degrade plastic (Cappitelli and Sorlini, 2008; Moharir and Kumar, 2019). The polyacrylic polymers used here were found to be mineralized by white-rot fungi (Sutherland et al., 1997) which are capable of degrading the most recalcitrant biological polymers (i.e., lignin) (Kirk et al., 1978). Nevertheless, for our experimental setup, we do not use white- rot fungi, and we also do not see a confounding effect by the potential carbon utilization from polyacrylic fibers by our fungi. We provided sufficient, easily available organic C for the fungi here, such that any utilization of C from the fibers should have played a minor role.

Microfiber addition alone, in the absence of fungi, had no significant effect ($p > 0.05$) on the percentage of WSA. This suggests that effects of microfibers on soil aggregation require the presence of soil biota (Lehmann et al., 2019a); the latter are necessary to build soil aggregates, in our experiment fungi, but incubating soil with just the microfibers evidently had no deleterious direct (physical) effects on WSA.

Microfiber addition negated the positive effect of rising temperature on WSA in the case RLCS 01, RLCS 07 and RLCS 08. Nonetheless, the effects of these two factors were not additive. Regarding RLCS 07 and RLCS 08*,* effects of rising temperature even turned out to be negative in the presence of microfibers, leading to greater loss in the percentage of WSA than microfiber alone. We currently do not know what caused this effect. This was most

likely because rising temperature led to faster fungal growth together with faster decomposition of soil organic matter increasing the production of binding agents for soil aggregates, thus resulting in more aggregate disintegration (Rillig et al., 2002) and formation under rising temperature, in other words a greater turnover of macroaggregates. Microfibers can be entrapped within macroaggregates (Bläsing and Amelung, 2018), perhaps leading to less stable macroaggregates. Thereby, when higher temperatures benefited WSA, microfiber fibers might be incorporated into newly forming macroaggregates, resulting in less stable aggregates. Moreover, for soil with RLCS 06, the negative effects of rising temperature disappeared in the presence of microfibers, it is possible that RLCS 06 accelerated decomposition of soil organic matter under gradually elevated temperature, thus fewer microfibers became incorporated into aggregates when the effect of gradually elevated temperature on WSA was negative; this could explain why the negative effect of gradually elevated temperature on WSA disappeared in the presence of microfiber.

In our study, the effect of microfibers also depended on temperature change patterns. We hypothesized that the effect of microfibers on WSA depended on how WSA responded to the temperature increase pattern.

An increase in FDA hydrolysis activity caused by rising temperature was shown previously (Sinsabaugh et al., 2008; Baldrian et al., 2013), indicating that warming increased fungal activity. Nevertheless, the differences of FDA between rising temperature and ambient temperature are not statistically clear except for soil with RLCS 08. We suggest that even small changes in FDA might cause large changes in the percentage of WSA, or that fungal effects not captured by FDA are important. The rising temperature might enhance the expression of traits that benefit formation and stabilization of aggregates by certain fungi: RLCS 01, RLCS 07 and RLCS 08. Such traits include the stability and longevity of hyphae, entanglement potential of soil particles (Lehmann and Rillig, 2015; Rillig et al., 2015) and also higher secretion rate of protein and metabolic products in warmer conditions (Papagianni, 2004).

Further research is needed to measure fungi-caused decomposition and fungal biomass density, in order to decouple the negative effect and positive effect of fungi on soil aggregation formation. Therefore, we should develop the method which could distinguish the plastic C from soil C, such as using ^{13}C- labeled microplastic (Zumstein et al., 2018).

2.6 Conclusion

In our study, plastic microfibers might eliminate the positive effect of temperature on soil aggregation, and could even lead to greater losses in the percentage of WSA. Thus, we emphasize the importance of considering the potentially strong decrease due to interactive effects between microplastics and global warming. Our study lends further support to general findings of prior research on the interactive effects of environmental factors on soil function (García et al., 2018; Tekin et al., 2018), suggesting strongly that global change effects should be analyzed not only as single factors but also in combination.
We found that sensitivities to the environmental factors differed among fungal species, and thus our study opens the door to the examination of the behavior of fungal communities when exposed to this combination of environmental factors.

2.7 Author Contributions

Y.L. designed and performed the research, conceived and performed the data analyses; Y.L. A.L., M.B. and M.R wrote the manuscript. All authors contributed to the final version of the manuscript.

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3. EFFECT OF MICROPLASTIC FIBERS ON SOIL AGGREGATION AND ENZYME ACTIVITIES ARE ORGANIC MATTER DEPENDENT

Yun Liang, Anika Lehmann, Gaowen Yang, Eva F Leifheit and Matthias C Rillig

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3.1 Abstract

Microplastic as an anthropogenic pollutant accumulates in terrestrial ecosystems over time, threatening soil quality and health, for example by decreasing aggregate stability. Organic matter addition is an efficient approach to promote aggregate stability, yet little is known about whether microplastic can reduce the beneficial effect of organic matter on aggregate stability. We investigated the impacts of microplastic fibers in the presence or absence of different organic materials by carrying out a soil incubation experiment. This experiment was set up as a fully factorial design containing all combinations of microplastic fibers (no microplastic fiber addition, two different types of polyester fibers, and polyacrylic) and organic matter (no organic matter addition, *Medicago lupulina* leaves, *Plantago lanceolata* leaves, wheat straw, and hemp stems). We evaluated the percentage of water-stable aggregates (WSA) and activities of four soil enzymes (β-glucosidase, β-D-celluliosidase, N-acetyl-b-glucosaminidase, phosphatase). Organic matter addition increased WSA and enzyme activities, as expected. In particular, *Plantago* or wheat straw addition increased WSA and enzyme activities by 224.77% or 281.65 % and 298.51% or 55.45%, respectively. Microplastic fibers had no effect on WSA and enzyme activities in the soil without organic matter addition, but decreased WSA and enzyme activities by 26.20% or 37.57 % and 23.85% or 26.11%, respectively, in the presence of Plantago or wheat straw. Our study shows that the effects of microplastic fibers on soil aggregation and enzyme activities are organic matter dependent. A possible reason is that Plantago and wheat straw addition stimulated soil aggregation to a greater degree, resulting in more newly formed aggregates containing microplastic, the incorporated microplastic fibers led to less stable aggregates, and decrease in enzyme activities. This highlights an important aspect of the context dependency of microplastic effects in soil and on soil health. Our results also suggest risks for soil stability associated with organic matter additions, such as is common in agroecosystems, when microplastics are present.

3.2 Introduction

Microplastics as a group of anthropogenic contaminants are pervasive and persistent. Microplastic is widely studied in marine ecosystems (Eriksen et al., 2014; Bergmann et al.,

2015; Jambeck et al., 2015), and only in recent years has attention shifted to terrestrial ecosystems (Rillig, 2012; Bläsing and Amelung, 2018; de Souza Machado et al., 2018a; Lozano and Rillig, 2020; Zhou et al., 2020). In fact, microplastic has been proposed as a new global change factor (Rillig and Lehmann, 2020). We adopted the definition that m icroplastics are plastics with size smaller than 5 mm (Moore, 2008; Barnes et al., 2009), with various shapes (e.g., fiber, fragment, film) and polymer types (e.g., polyester, polyethylene, polyacrylic, polypropylene), which are intentionally produced (e.g., microplastic beads in cosmetics) or fragmented into micro-sized plastics by natural or anthropogenic factors, such as photooxidation (Yakimets et al., 2004; Gewert et al., 2015), microbial degradation (Zettler et al., 2013; Moharir and Kumar, 2019) or ploughing (Hann et al., 2016).

In microplastic polluted soil, microplastic fibers are a dominant shape (Singh et al., 2020; Zhou et al., 2020). Microplastic fibers derived from textiles are commonly discovered in wastewater (Pirc et al., 2016; Athey et al., 2020), they can span a length range of 0.3- 25.0 mm. These microplastic fibers derived from textiles are therefore present in sludge and biosolids, which are applied on agricultural fields as fertilizer (Henry et al., 2019; Crossman et al., 2020; Zhang et al., 2020), likely leading to the accumulation of microplastic fibers in agricultural soils (Corradini et al., 2019; van den Berg et al., 2020). It is estimated that 1.56 × 1014 microplastic particles could enter the soil and other natural environments through sludge per year in China alone (Li et al., 2018). The majority of those fibers are made of polyester and polyacrylic. Moreover, atmospheric deposition of microplastic fibers is an important source of soil contamination, as hundreds of particles are deposited from the atmosphere per square meter per day (Cai et al., 2017; Dris et al., 2017; Brahney et al., 2020). Further anthropogenic activities, such as tillage, and also movement by soil animals can accelerate the incorporation of microplastic fibers into the soil (Huerta Lwanga et al., 2017; Rillig et al., 2017).

Microplastic fibers have been shown to influence soil quality and health by being detrimental to soil aggregate stability (de Souza Machado et al., 2018b; Lehmann et al., 2019a) and altering microbial activity (Liu et al., 2017; de Souza Machado et al., 2018b; Huang et al., 2019). Stability of soil aggregates, a fundamental soil physical property, is crucial to resist erosion, support water infiltration, water retention, aeration, and fertility (Bryan, 1968; Tisdall and Oades, 1982; Boix-Fayos et al., 2001; Li et al., 2016). Soil is a dynamic and complex system, and in particular the stability of soil aggregates is influenced by many factors including organic matter input, soil texture, clay mineralogy, and microbial

populations (Seta and Karathanasis, 1996; Bossuyt et al., 2001; Wagner et al., 2007). Among these, organic matter is one of the most important factors determining aggregation (Abiven et al., 2008). The addition of organic matter promotes stable aggregation for example by stimulating microbial growth and metabolism, leading to increases in microbially derived metabolites such as polysaccharides and proteins, which, together with plant-derived polysaccharides act as glueing agents that facilitate aggregate stabilization (Tisdall, 1994; Wright and Upadhyaya, 1998; Caesar-Tonthat, 2002). The degradability of organic matter / litter is an inherent property of the material, leading to different breakdown products being released. Therefore, the effects of organic matter on aggregation varied among different types of organic matter (Abiven et al., 2008). We have previously shown that under soil conditions favorable to the formation of aggregates, microplastic fiber addition could reduce the stability of aggregates (Liang et al., 2019), because newly formed aggregates are likely to have incorporated microplastic fibers which can reduce stability of aggregates, likely by introducing fracture points. Given that organic matter addition facilitates the formation of aggregates, we assume that microplastic fibers have a more detrimental effect on WSA when organic matter is added, and that the magnitude of the effect depends on the type of organic matter.

Soil enzyme activity is key to biological processes, driving nutrient cycles in terrestrial ecosystems. Enzyme activity as an important indicator of microbial activity is frequently altered by microplastics (de Souza Machado et al., 2018b; Liang et al., 2019). As microplastic fibers tend to reduce bulk density and increase soil porosity (de Souza Machado et al., 2018b; Zhang et al., 2019a), microplastic fibers are expected to improve aeration, and thus increase enzyme activity. Moreover, microplastics themselves as organic carbon might introduce an artificial carbon source (Rillig, 2018), possibly influencing enzyme activity by being a potential substrate. However, the overall outcome of the interactive effects of microplastic fibers and organic matter on aggregates and enzyme activity remains unknown, and is the subject of our investigation here.

We carried out a soil incubation experiment with all combinations of 3 types of microplastic fibers and 4 types of organic matter. We hypothesize: 1) soil aggregation will be decreased by microplastic fiber, but effects will depend on the type of organic matter; 2) microplastic fibers will increase soil enzymatic activities.

3.3 Materials and methods

3.3.1 Microplastic fiber

Two polyester products (polyester1, PE1: Rope Paraloc 137 Mamutec polyester white, item number, 8442172, Hornbach.de, diameter: 0.03mm, density: 1.45 g cm-3; polyester 2, PE2: Dolphin Fine 5 x 100 g Himalaya Knitting Wool, Baby Wool, 500 g Super Bulky Wool, diameter: 0.008mm, density: 1.37 g cm-3) and one polyacrylic, PA (100% acrylic "Bravo" yarn, (schachenmayr.com), diameter: 0.026mm, density: 1.31 g cm-3) product were used in this study (Appendix Figure A3.1). These types of plastics are widely used in textiles (Carney Almroth et al., 2018), and have previously been shown to decrease soil aggregation (de Souza Machado et al., 2018b).We produced microplastic fibers by manually cutting them into fragments of approximately 5 mm in length, the average lengths of PE1, PE2, and PA are 4.56±0.94 mm, 4.20±1.37 mm, 4.05±0.1.14mm, respectively, the size distributions are given in Appendix Figure A3.2. The estimated particle numbers of PE1, PE2, and PA are 193, 2.9×10^4, 2.87×10^3 items g^{-1} dry soil, respectively These lengths were chosen to match the criteria of microplastic upper limit and the general size range of secondary microplastic fibers produced by washing of clothes made from synthetic fibers (Pirc et al. 2016). We rinsed the fibers with tap water for 5min to remove soluble chemicals, then dried them at 60 °C for 24 h, and subsequently microwaved them for 3 min to reduce any microbial populations adhering to the material. The microwaving does not alter the physical appearance of the treated microplastic products (de Souza Machado et al. 2018b). The microplastic fibers were mixed into the soil at a concentration of 0.3% (w/w), towards the upper limit of concentrations used in a previous study (de Souza Machado et al., 2018b), such concentration was also applied in other study (Zhang et al., 2019b). The concentration 0.3% we used is within the contamination range in a plastic industrial area, the soil in which contained 0.03% to 6.7% of microplastic (Fuller and Gautam, 2016).

3.3.2 Organic matter

We chose four different types of organic matter: *Medicago lupulina* leaves, *Plantago lanceolata* leaves, wheat straw (MultiFit, Item no.: 1008159, Krefeld, Germany), and hemp stems (REAL NATURE, Item no.: 1259176, Krefeld, Germany) (Appendix Figure A3.3). *Medicago lupulina* and *Plantago lanceolata* leaf material were chosen as typical species from the local grassland, which were collected from plants previously grown in our greenhouse,

wheat straw was chosen as the type of organic matter widely applied in agriculture for soil amendments, hemp was used to represent woody plant litter. *Medicago*, *Plantago*, straw and hemp formed a gradient of litter quality with C:N ratios as 12.85±0.11%, 14.76±0.29%, 133.03±2.18%, 153.04±0.70, respectively; the decomposition of *Medicago* was the fastest, followed by *Plantago*, and straw, with hemp being the slowest to decompose (Appendix Figure A3.4). Before adding organic matter to our experiment, we ground the material using a blender (Philips Pro Blend 6 RD, Germany) and sieved to keep size between 0.5 mm and 2 mm. We added organic matter with a concentration of 0.8% (w/w) to our test systems. The concentration of 0.8% was below the saturation level of organic matter addition in our test soil (Appendix Figure A3.5).

3.3.3 Experimental design

This experiment was set up as a fully factorial design and contained all combinations of microplastic fibers (no microplastic fiber addition (No mf), PE1, PE2, PA) and organic matters (no organic matter addition (No OM), *Medicago*, *Plantago*, wheat straw, hemp stems), resulting in 20 treatments including the controls. Each treatment had 8 replicates for a total of 160 experimental units.

3.3.4 Soil incubation

Fresh soil was collected from a local grassland (Berlin, Germany) with a sandy loam texture, an Albic Luvisol (Rillig et al., 2010). Soil was sieved < 0.5 mm in order to reduce the amount of larger soil aggregates, thus intensifying the effect of organic matter addition on aggregate formation. Reducing aggregates beforehand is commonly used to measure macroaggregate formation in laboratory incubations (De Gryze et al., 2005). We mixed 20 g of the dry soil with 60 mg of microplastic fibers by steel spoon for 3 minutes, achieving a homogeneous distribution of fibers as no obvious fiber clusters could be observed by eye, then we mixed the prepared soil with 160 mg of organic matter of the different types for 30s which is sufficient time to achieve an even distribution of organic matter. We applied the 3.5 minutes of mixing time to the soil without microplastic or organic matter addition aiming for the same level of disturbance. The soil mixture was transferred to a 50 ml falcon tube by using a steel spoon, the soil mixture was placed into the tube carefully in order to maintain the distribution of microplastic fiber in soil we achieved after mixing. Then we slowly wetted soil with distilled water by injecting water into soil by using a syringe, the water passively spread

throughout our test system. We kept water content at 60% water holding capacity. Tubes were then closed with a hydrophobic vented cap to allow gas exchange. We centrifuged all tubes with soils at 100 rpm/s for 1 min to minimize any cracks in the soil (Brackin et al., 2013), and then incubated them at 25 °C in the dark for 42 days, we assumed organic matter might achieve an intensive effect on aggregate stability after 42 days (Abiven et al., 2008).

3.3.5 Aggregate stability measurement

To measure soil aggregate stability, we followed the protocol by Kemper and Rosenau (2018): the air-dried soils were sieved through a 2 mm sieve and 4.0 g of soils were placed into sieves for capillary rewetting in deionized water for 5 min. We used 0.25 mm sieves to test the stability of the soil fraction > 0.25 mm (macroaggregates) against water as a disintegrating force. For the test, sieves carrying the soil samples were placed in a wet-sieving machine (Eijkelkamp, Netherlands) and moved vertically (stroke = 1.3 cm, 34 times min^{-1} for 3 min. The fractions left on the sieves were dried at 60 °C for 24 h. After weighing the dry fractions, sand particles and organic debris larger than 0.25 mm were extracted from the fractions as coarse matter. The calculation of percent water-stable aggregates (WSA) was: % WSA= （water stable aggregates - coarse matter) / (4.0 g - coarse matter).

3.3.6 Enzyme activity measurement

To assess the ability of the microbial community to acquire nutrients, we measured activities of β-glucosidase (cellulose degradation), β-D-celluliosidase (cellulose degradation), N-acetyl-b-glucosaminidase (chitin degradation), and phosphatase (organic phosphorus mineralisation) (Delgado-Baquerizo et al., 2017). These enzymes are key for microbes to acquire C, N and P, and are thus at least partially indicative of the function of the microbial community in terms of organic matter processing and decomposition (Waldrop et al., 2000). Soil enzymatic activity was determined using a high throughput microplate assay, according to Jackson et al. (2013). Activities of above enzymes were measured using p-nitrophenyl (pNP) -linked model substrates : pNP-β-D-glucopyranoside (Sigma no. N7006), pNP-β-D-cellobioside (Sigma no. N5759), pNP-N-acetyl-β-D-glucosaminide (Sigma no. N9376), pNP- phosphate disodium salt hexahydrate (Sigma no. 71768), respectively. Briefly, 3.5 g of frozen stored (-20°C) soil of each sample was placed in a sterile 50 ml centrifuge tube and mixed with 10 ml of 50 mM acetate buffer, and then the mixture was vortexed for 30s to produce a soil slurry. After vortexing, the soil slurry was transferred to 96-well microplates. Reaction mixtures in each

cell contained 0.150 mL soil slurry and 0.150 mL substrate dissolved in 50 mM sodium acetate buffer (pH 5.0). Reaction mixtures were incubated at 25°C for 2 - 4 h. After incubation, microplates were centrifuged at 3000 rpm for 5min, then 0.100 ml of suspension was transferred into a new microplate and mixed with 0.200 mL 0.1 M NaOH to stop the reaction. Absorbances were determined spectrophotometrically at 410 nm using a microplate reader (BioRad, Benchmark Plus, Japan). Enzyme activity was defined as the amount of released µmol of p-nitrophenol per gram of dry soil per hour.

3.3.7 Statistical analysis

All statistics were conducted in R 3.5.3 (R Core Team, 2017). We analysed the effect size of microplastic fibers on WSA and enzyme activity using the package "dabestr" (Ho et al., 2019) to generate unpaired mean differences and 95% confidence interval (CI) by a bootstrapping approach (5000 iterations). This type of analysis estimates the magnitude and precision of an effect. To support our findings, we additionally applied two-way ANOVA by using generalized least square models in the "nlme" package (Pinheiro et al., 2020). The plots were created with the graphic package 'ggplot2' (Wickham, 2016).

3.4 Results

3.4.1 Stability of soil aggregates

All types of organic matter increased WSA substantially compared to the control, with wheat straw having the most positive effect on WSA [30.7% (95%-CI: 26-35.1)], followed by *Plantago* [24.4% (95%-CI: 20.6-29.1)], hemp stems [24.2% (95%-CI: 19-28.4)], and *Medicago* [16.4% (95%-CI: 11-22.8)].

The effects of microplastic fibers on WSA strongly depended on the type of added organic matter (Figure 3.1, Table 3.1). Microplastic fibers had neutral effects on WSA in soil without organic matter addition and soil with *Medicago* addition. By contrast, all types of microplastic fibers exerted negative effects on WSA in soil with *Plantago* and wheat straw; PE2 had negative effects in soil with hemp stem. The most negative effect of microplastic fibers on WSA was found in soil with PE1 and *Plantago* [-13.3%, (95%-CI: -18.7 to -6.55)], followed by PE1 and wheat straw [-10.9%, (95%-CI: -16.2 to -6.43)]. Generally, microplastic fibers were more detrimental for WSA in soils with added organic matter, while organic matter addition was more beneficial to WSA (Figure 3.2).

Table 3.1 ANOVA results for the effects of organic matter, microplastic fibers and the interaction of these factors on the stability of soil aggregates (WSA), β-Glucosidase (BG) activity, cellobiohydrolase (CB) activity, phosphatase (Phos) activity and N-acetyl-glucosaminidase (NAG) activity.

Treatment	DF	WSA		BG activity		CB activity		Phos activity		NAG activity	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Organic matter	4	141.29	**<.0001**	74.83	**<.0001**	95.98	**<.0001**	71.84	**<.0001**	584.18	**<.0001**
Microplastic fiber	3	11.38	**<.0001**	4.78	**<.01**	3.20	**<0.05**	2.88	**<0.05**	1.82	0.15
Microplastic fiber: Organic matter	12	2.55	**<.01**	0.61	0.83	0.93	0.52	0.60	0.84	0.98	0.47

$p < 0.05$ was considered significant and marked in bold. Degrees of freedom (DF), F- and p-value for each variable are presented.

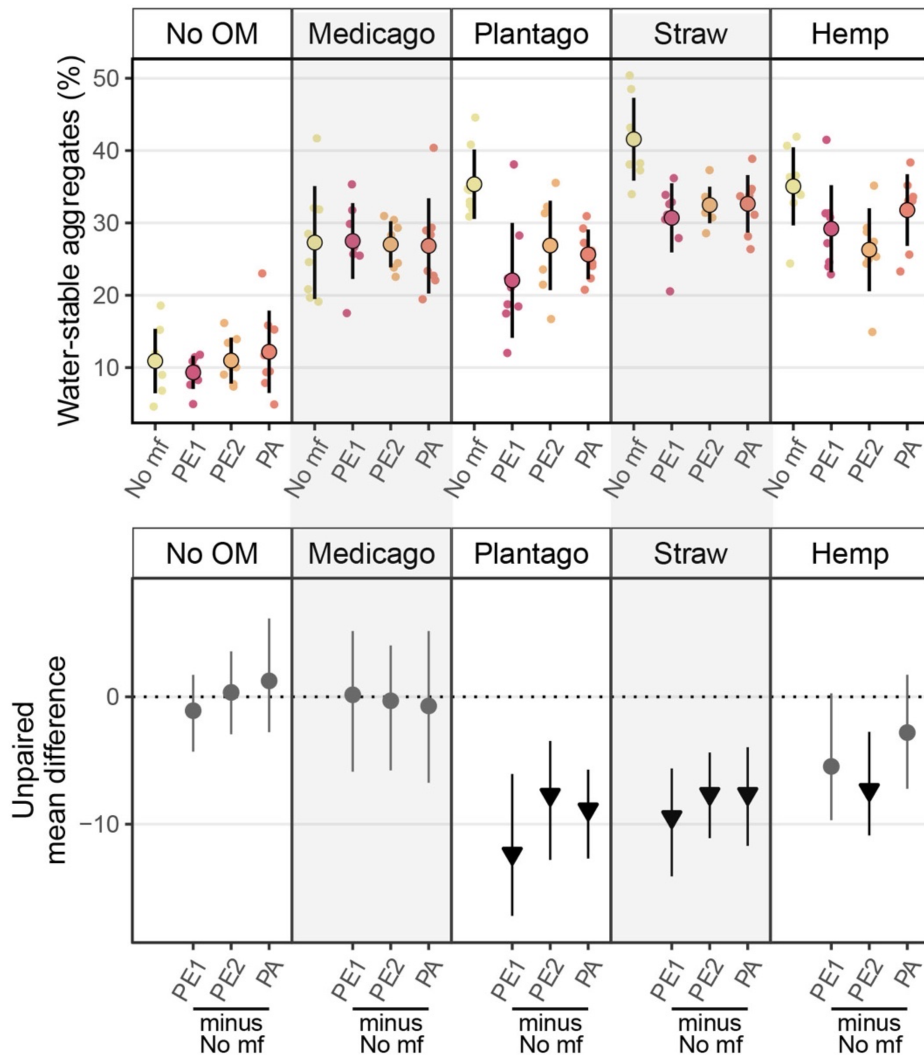

Figure 3.1. Effects of microplastic fibers on soil water stable aggregates (WSA in %) with the addition of different types of organic matter. The upper panel shows the raw data of water stable aggregates, data distributions are aligned with corresponding mean and standard deviation (n = 8 for each treatment). The lower panel shows the unpaired mean differences of the microplastic fiber addition and control under different organic matter addition. Circles and triangles represent the effect size mean (unpaired mean; effect magnitude) and the vertical lines the corresponding confidence intervals (effect precision). Negative (arrow head down) effect sizes and corresponding CIs of treatment compared to control are depicted in black while neutral effects (circle) are colored in gray; neutral effects occur when the CIs overlap the dashed zero line (line of no effect). “No OM” represents no organic matter addition, “No mf” represents no microplastic fiber addition.

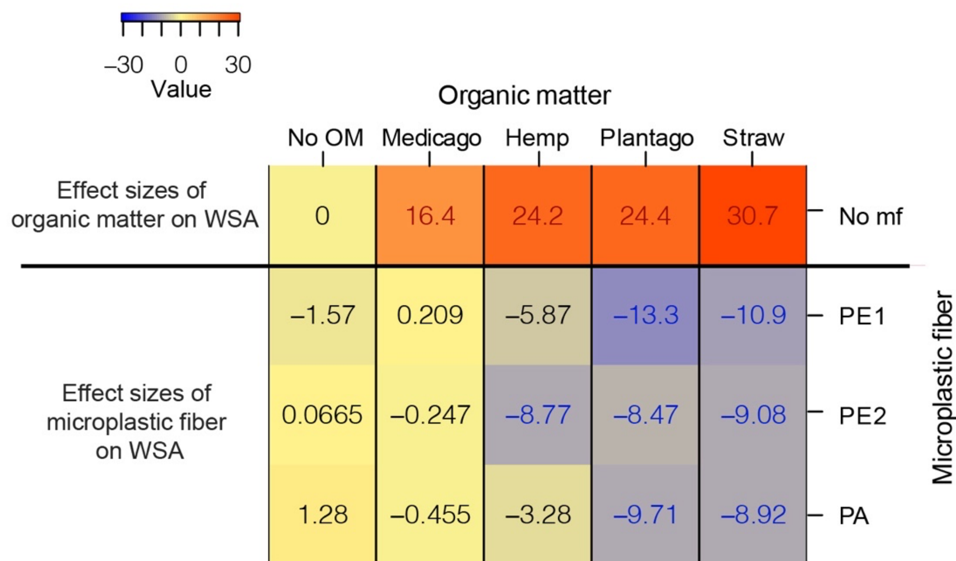

Figure 3.2. The heatmap of the effect sizes of microplastic fiber and organic matter on WSA. The effect sizes of organic matter on WSA were the unpaired mean differences of organic matter addition alone and control ($WSA_{organic\ matter} - WSA_{No\ OM}$). The effect sizes of microplastic fiber are the unpaired mean differences of the microplastic fiber addition and control with the addition of the different types of organic matter.($WSA_{organic\ matter_microplastic\ fiber} - WSA_{organic\ matter_No\ mf}$) The blue text represents the statistically significant negative effect of microplastic fiber on WSA, the red text represents the statistically significant positive effect on WSA. “No OM” represents no organic matter addition, “No mf” represents no microplastic fiber addition.

3.4.2 Enzyme activities

Organic matter addition stimulated enzyme activities, while microplastic fibers decreased enzyme activities in some cases. The effects of microplastic fibers on enzyme activities were neutral in soil without organic matter addition, while with organic matter addition, negative effects of microplastic fibers appeared (Figure 3.3, Table 3.1). We found the highest loss of β-Glucosidase activity in soil with PE1 and *Plantago* [-1.64 μmol pNP g^{-1} h^{-1}(95%-CI: -0.03 to -3.27)], cellobiohydrolase activity in soil with PE2 and hemp stem [-0.19 μmol pNP g^{-1} h^{-1}(95%-CI: -0.07 to -0.35)], phosphatase in soil with PE2 and wheat straw [-1.22 μmol pNP g^{-1} h^{-1}(95%-CI: -0.47 to -1.96)], N-acetyl-glucosaminidase in soil with PA and wheat straw [-0.65 μmol pNP g^{-1} h^{-1}(95%-CI: -0.07 to -1.24)].

We found a similar relationship between effects of microplastic fibers in the presence of different types of organic matter and effects of organic matter on β-Glucosidase activity as we found in WSA; meaning, the more positive an effect a specific organic material, the more detrimental was the impact of added microplastic fibers (Figure 3.4).

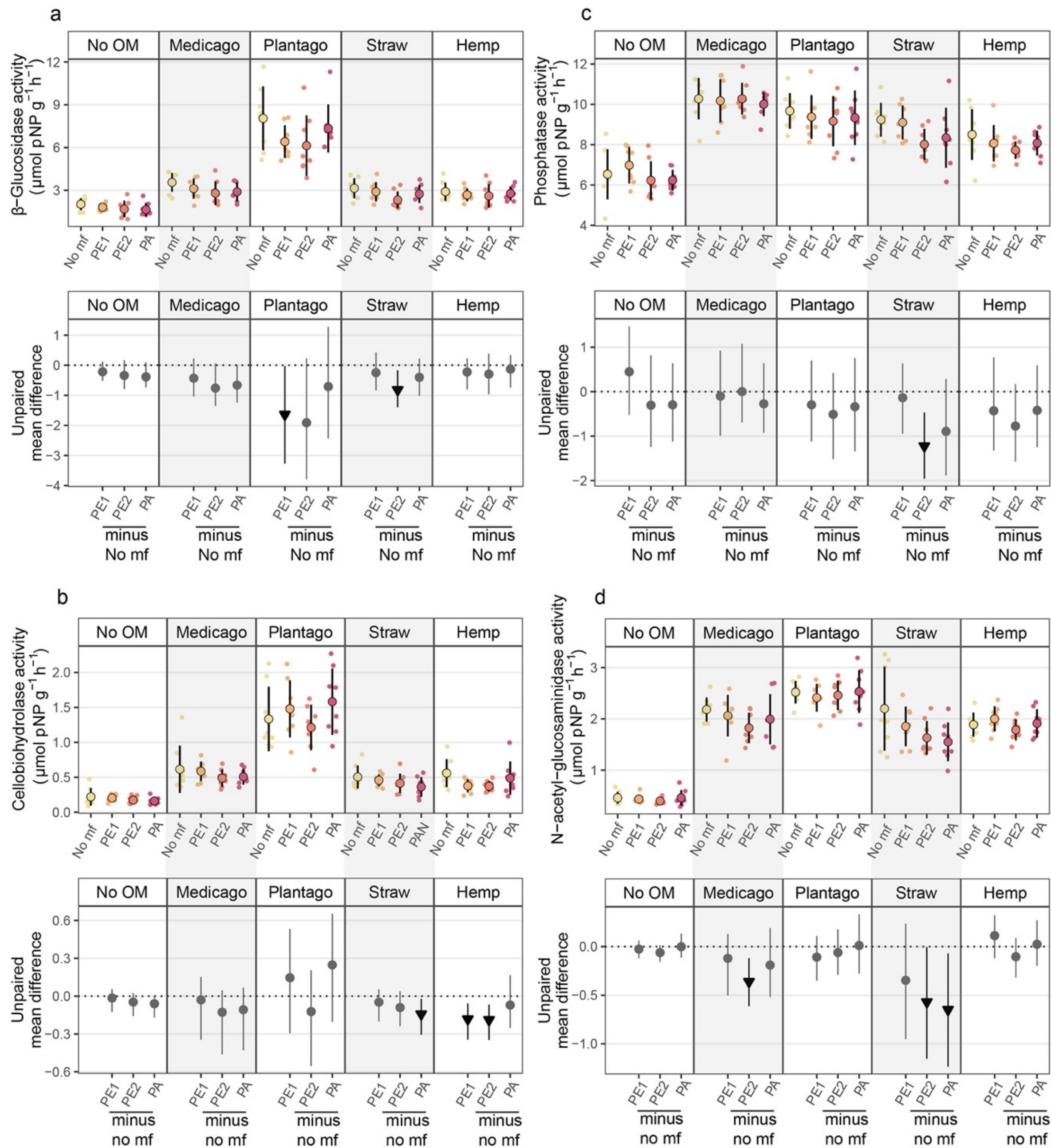

Figure 3.3. Effects of microplastic fibers on β-Glucosidase activity (a), cellobiohydrolase activity (b), phosphatase activity (c) and N-acetyl-glucosaminidase activity (d) in the presence of different types of organic matter. The upper panel shows the raw data of enzyme activity, data distributions are aligned with corresponding mean and standard deviation (n = 8 for each treatment). The lower panel shows the unpaired mean differences of the

microplastic fiber addition and control with different types of organic matter addition. Circles and triangles represent the effect size mean (unpaired mean; effect magnitude) and the vertical lines the corresponding confidence intervals (effect precision). Negative (arrow head down) effect sizes and corresponding CIs of treatment compared to control are depicted in black while neutral effects (circle) are shown in gray; neutral effects occur when the CIs overlap the dashed zero line (line of no effect). "No OM" represents no organic matter addition, "No mf" represents no microplastic fiber addition.

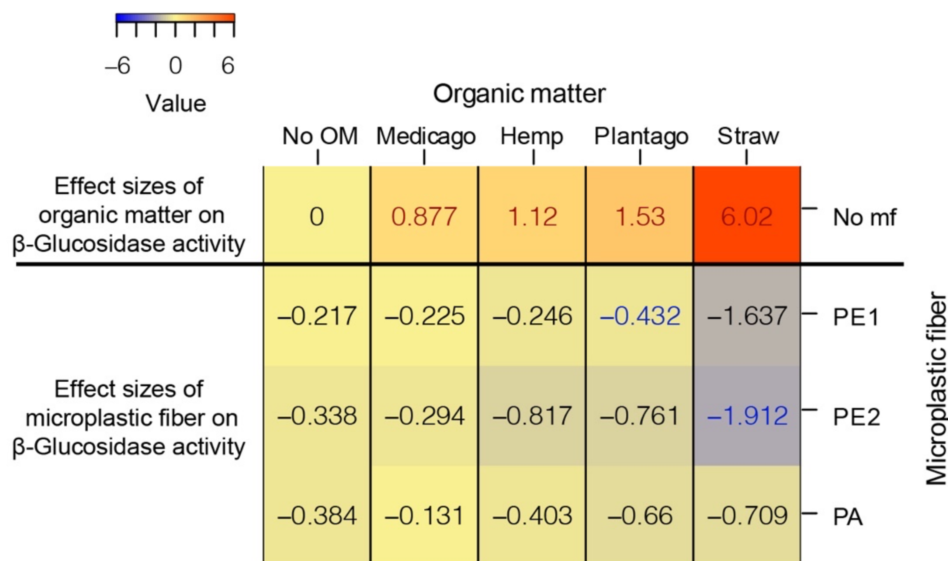

Figure 3.4. The heatmap of the effect sizes of microplastic fiber and organic matter on β-Glucosidase activity. The effect sizes of organic matter on β-Glucosidase activity were the unpaired mean differences of organic matter addition alone and control (β-$_{\text{Glucosidase activityorganic matter}}$ - β-Glucosidase activity$_{\text{No OM}}$). The effect sizes of microplastic fiber are the unpaired mean differences of the microplastic fiber addition and control with the addition of the different types of organic matter (β-Glucosidase activity$_{\text{organic matter_microplastic fiber}}$ - β-Glucosidase activity$_{\text{organic matter_No mf}}$). The blue text represents the statistically significant negative effect of microplastic fiber on β-Glucosidase activity, the red text represents the statistically significant positive effect on β-Glucosidase activity. "No OM" represents no organic matter addition, "No mf" represents no microplastic fiber addition.

3.5 Discussion

3.5.1 Effects of microplastic fibers on the stability of soil aggregates (WSA)

As we hypothesized, microplastic fibers reduced the stability of aggregates in the presence of specific types of organic matter, with the magnitude of the microplastic fiber effects dependent on the type of organic matter. This was likely because the addition and subsequent microbial processing of organic matter accelerated aggregation, which in turn led to more microplastic fibers being incorporated into newly formed aggregates. The concentration of microplastic fibers in aggregates was previously observed to have increased with the addition of one type of organic matter addition (Zhang and Zhang, 2020). In addition, the increased incorporation of microplastic fibers into soil aggregates might produce fracture points, therefore reducing the stability of the coarse, sandy soil that we used here. Given that the effects of organic matter on aggregation varied among different types of organic matter, the amount of increased incorporation of fibers into aggregates might depend on the type of added organic matter; this helps explain the phenomenon that microplastic fibers generally exerted more negative effects on WSA in soil to which a type of organic matter was added that favored soil aggregation.

In our study, microplastic fibers had no effects on WSA in soil without added organic matter. The sandy loam soil we used had limited intrinsic potential to form aggregates, thus there must have been only relatively few microplastic fibers that were incorporated into aggregates, resulting in no effects of microplastic fibers in soil without added organic matter. We assumed organic matter stimulated microbial activity, accelerating the formation of aggregates and the integration of fibers into those aggregates. A similar result was found in a previous study (Lehmann et al., 2019b): microplastic fiber had no effects on aggregate stability in sterile soil, while decreased aggregate stability in soil with a microbial community, indicating that microorganisms mediate effects of microplastic fibers on soil aggregate stability (Forster, 1990; Lehmann et al., 2017). A contrary result was found in a study using clayey non-sterile soil, in which polyester fiber increased macroaggregates (Zhang et al., 2019b). Soil mineralogy and clay content play important roles in the formation and stability of aggregates (Seta and Karathanasis, 1996), microplastic fibers may have different effects on soil aggregates in soils with different textures. Further studies are needed to explore the interaction between microplastic and soil minerals.

Our WSA results also reveal that predictions based on just the type of microplastic fibers alone would have failed: effects are dependent on the type of organic matter available for microbial processing - and thus on the rate of soil aggregate formation -, and as such our study has uncovered an important aspect of context dependency of microplastic effects in

soils. However, there is a limitation to our study. The temporal effects of organic matter input on soil aggregation are not covered in our study design. *Medicago* had the lowest C:N ratio, thus had the fastest decomposition rate. Therefore, we expected the greatest increase of WSA in soil amended with *Medicago* alone, and also the greatest loss in WSA caused by microplastic fibers in soil amended with *Medicago*. Nevertheless, neither was observed in our study. We assumed the *Medicago* as a rapidly degraded organic matter exhibited the maximum aggregate stability before we harvested the experiment, then the aggregate began to break down as the binding agents were decomposed (Abiven et al., 2008). Our findings could be relevant for agroecosystems: wheat straw is widely used as an organic amendment in agriculture due to the benefits it offers, such as increasing sequestration of soil organic carbon and improving soil structure (Han et al., 2018; Zhao et al., 2019). However, as agricultural fields are prone to microplastic contamination, our study suggests that this can lead to unforeseen effects of straw additions on soil properties; an assertion that should now be explicitly tested in the field.

3.5.2 Effects of microplastic fibers on enzymatic activities

Contrary to our hypothesis, microplastic fibers did not increase enzyme activities, but had even negative effects on enzyme activities with the addition of specific types of organic matter. The results depended on the microplastic type, but the pattern was not consistent across the organic matter treatments. PE2 decreased enzyme activities more frequently than PE1 and PAN, which might be attributed to the highest particle numbers of PE2 in each experimental unit. Moreover, those negative effects were more likely to appear in the presence of straw. This again emphasizes the context dependency of microplastic impacts on the soil environment. Such negative effects of microplastics are also found in other studies, as microplastics reduce soil nutrient levels (Yu et al., 2020). We do not know what caused this negative response in our study, but microplastic fibers might have caused shifts in the microbial community via changing soil physical properties(de Souza Machado et al., 2018b), releasing additives (Widén * et al., 2004; Hahladakis et al., 2018) that were not water-soluble (fibers were washed before being mixed in the soil) that migrated into the soil (Kim et al., 2020), or serving as microbial habitats (Huang et al., 2019; Zhang et al., 2019c); the enzymatic activities could have changed as a consequence of such community shifts.

Microplastic fibers had negative effects on enzyme activities in the presence of organic matter, which could lead to decreased nutrient uptake into microbes and mineralization rates.

The restricted nutrient transfer from organic matter to microbes could change the microbial community in the long term, potentially resulting in altered soil functions and processes (Waldrop et al., 2000) . Additionally, assuming an accumulation of microplastic fibers in the future, decreased enzyme activities could lead to a reduction of plant available nutrients and thus ultimately diminish agricultural productivity or crop quality (Paz-Ferreiro et al., 2014).

Moreover, the decreased nutrient uptake could lead to decreased microbial biomass and metabolites, which are important for accumulating stabilized C in soil (Cotrufo et al., 2013; Cenini et al., 2015). Nevertheless, decreased enzyme activities accompanied by decreased transformation of organic matter could also lead to less C loss, the eventual fate of added C could not be predicted from our study due to the scope of our work here. Further studies should target long-term C dynamics of added organic matter, to enable prediction of the potential effects of microplastic fibers on C sequestration.

Though soils received different types of microplastic fibers, which varied in diameters, particle numbers per experimental unit, and probably varied in their additives, we did not find a consistent pattern of the different microplastic fibers across our response variables. In terms of WSA, as a previous study found microplastic fibers decreased WSA with increasing concentrations of microplastic fibers (de Souza Machado et al., 2018b), we assume the decreases in WSA already reached the upper limit response of soil to microplastic fibers in our study, resulting in no obvious different effects on WSA among the types of microplastic fibers.

In terms of enzyme activities, we found the decrease in enzyme activities appeared randomly among microplastic fibers. We assumed microplastics needed a longer time to reveal their effects on the microbial community and thus on microbial function, such as accumulation of additives released from aged plastic over time (Bandow et al., 2017).

Microplastic fibers we produced had higher average length than that microplastic fibers found in the environment, with most studies finding fibers of shorter than 1mm(Cai et al., 2017; Zhou et al., 2018; Corradini et al., 2019). For our experimental work, it was impractical to manually cut fibers that small (but see Schmiedgruber et al., 2019; Frehland et al., 2020).

3.6 Conclusions

In our study, microplastic fibers affected soil aggregation by interfering with the formation of stable aggregates, with effects dependent on the type of added organic matter. It seems that

greater soil aggregation activity leads to increased opportunities for microplastic to interact with this biological process; this is very likely due to microplastic fibers becoming integrated into aggregates to an increasing degree, leading to subsequent destabilization of these structures by as yet unknown mechanisms. Effects of microplastic additions on soil processes have been variable, in part likely due to the plastic material itself, but our study points to soil properties, in particular soil organic matter, as another important variable contributing to the context dependency of such effects in terrestrial ecosystems.

3.7 Author Contributions

Y.L. and E.L. designed the research, Y.L. performed the research, conceived and performed the data analyses; Y.L., A.L., E.L., G.Y., M.R. wrote the manuscript. All authors contributed to the final version of the manuscript.

3.8 Acknowledgments

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4. SOIL CARBON PROTECTION BY AGGREGATES IS INFLUENCED BY TEMPERATURE AND MICROBIAL DIVERSITY

Yun Liang, Eva F. Leifheit, Anika, Lehmann, Matthias C. Rillig

4.1 Abstract

The occlusion of litter-derived organic carbon in soil aggregates is a crucial aspect of soil organic carbon (SOC) protection. This complex process is influenced by soil microbes and environmental factors, among which temperature is a key factor affecting SOC decomposition. We investigated the impacts of temperature (ambient temperature of 25°C or temperature increased by 2.5°C) and microbial diversity (low, medium and high) on the formation of aggregate-protected C by performing a soil incubation experiment. We evaluated the percentage of water stable aggregates, C loss, respiration and SOC in different soil fractions. Elevated temperature decreased the concentration of which in aggregates; microbial diversity was the most important for mineralization: low diversity had the highest C loss and the lowest amount of free light fraction (fLF). Though we found increased macroaggregates and amounts of macroaggregate-associated C in soil with low microbial diversity, the C concentration in macroaggregates was not increased. Our findings showed that decomposition was significantly influenced by microbial diversity in soil, which could be important in ecosystems with strong anthropogenic influence. We found that temperature mainly affected the formation of aggregate-protected C, which is important for predicting the stabilization of SOC in the long term under scenarios of global warming.

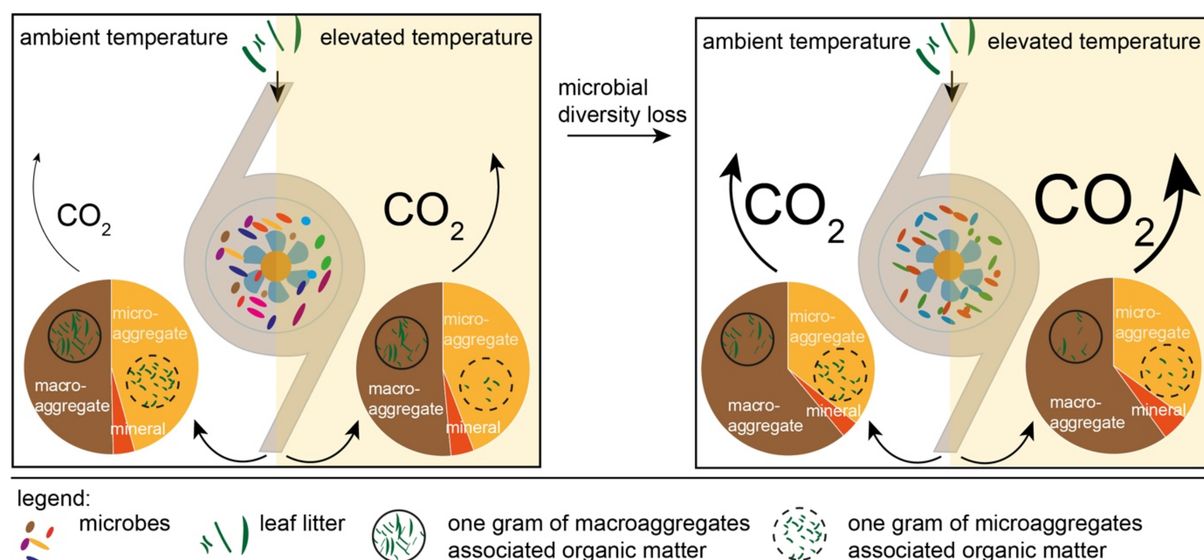

Figure 4.1 conceptual figure

4.2 Introduction

Litter input is the dominant source of soil organic carbon (SOC) in most ecosystems. During the process of litter decomposition, microbes take up nutrients and carbon compounds for growth and metabolism. Microbes secrete proteins and polysaccharides as metabolic by-products that can attach to mineral surfaces (Tisdall, 1994; Rillig et al., 2007). The adsorption of microbial detritus to mineral surfaces seems to be crucial in the formation of stable organo-mineral compounds (Kleber et al., 2007). Furthermore, organo-mineral associations facilitate soil aggregation, which is a key mechanism of SOC protection (Six et al., 2002; Von Lützow et al., 2008). The incorporation of the plant detritus, microbial-derived C and necromass into aggregates physically protects those against desiccation or predation, thus increasing stable SOC (Elliott et al., 1980; Six et al., 2006). Though macroaggregate occluded C will be quickly decomposed (Six et al., 2004; Six and Paustian, 2014), macroaggregates still play a crucial role in the occlusion of microaggregates and minerals (Six et al., 2004; King et al., 2019). The carbon in microaggregates or associated to minerals that is occluded, and thus protected inside macroaggregates, likely is the dominant stable SOC pool in most soils (Cotrufo et al., 2019). Therefore, macroaggregates are as important for C protection as are microaggregates.

Microbial diversity is vital in soil carbon cycling functions (Tardy et al., 2015). Many studies found positive relationships between microbial diversity and decomposition (Thormann, 2006; Baumann et al., 2013; Delgado-Baquerizo et al., 2016, 2020). Diverse microbial communities contribute to decomposition complementarity, leading to rapid decomposition and finally resulting in a higher production of gluing agents or proliferation of fungal hyphae that benefit soil aggregation (Tisdall et al., 1978). However, there are also diverging results showing a negative relationship between microbial diversity and decomposition (Griffiths et al., 2001; Deacon et al., 2006). The relationship between microbial diversity and decomposition has been studied for many years, but there is still a knowledge gap concerning how microbial diversity affects stable SOC formation, which is important for understanding SOC storage.

Temperature is the dominant factor that influences microbial SOC processing by stimulating microbial enzyme activity, leading to increased rates of decomposition, resulting in more CO_2 released by respiration (Hyvonen et al., 2007; Arora et al., 2013). Climate models predict that the mean annual global surface temperature will increase by 1- 3.5 °C until 2100 (Beckage et

al., 2018), while a global database of soil stock responses to warming suggests that one degree of warming could lead to 30-203 petagrams of C loss in the upper soil horizons (Crowther et al., 2016). However, decomposition stimulated by elevated temperature is restricted by substrate accessibility (Zimmermann et al., 2012; Fissore et al., 2013; Moinet et al., 2020). There is increasing consensus that physical protection of carbon compounds primarily drives the persistence of soil organic matter rather than the chemical recalcitrance of litter inputs (Kleber et al., 2011; Lehmann and Kleber, 2015; Moinet et al., 2020). Nevertheless, data on the influence of temperature on the stability or protection of SOC are limited. The available results suggest that aggregate-associated C may decrease due to warming (Poeplau et al., 2017; Guan et al., 2018).

We therefore performed an experiment with elevated temperature and microbial diversity levels as factors, to explore the effects of these factors on the formation of protected SOC. We used initially microbe- and carbon-free artificial soil to quantitatively assess the C distribution in different aggregate fractions after the decomposition of litter. We hypothesized: 1. Higher microbial diversity results in an increase in litter decomposition, accompanied by increased formation of microbial exudates that promote the formation of stable SOC compounds. We expected that higher microbial diversity will also lead to increased soil aggregate formation through the formation of microbially derived organic matter, organic polymers and fungal hyphae. 2. Increased temperature will increase litter decomposition, while it will also cause a loss in aggregate-protected SOC.

4.3 Materials and Methods

4.3.1 Experimental design

The experiment had a fully factorial design, with 6 unique combinations of the following treatments: 3 microbial diversity levels (low, medium, high), crossed with 2 temperature patterns (ambient and elevated temperature). Each treatment had 10 replicates for a total of 60 tubes.

4.3.2 Artificial soil

The artificial soils consisted of 7% kaolin clay, 5% quartz silt (< 250µm, carbonate free), 85.4% quartz sand (20-250µm, carbonate free) and 2.6% litter expressed as percentage of weight. We used finely ground leaves of *Acer platanoides* (C: 42.61%, N: 0.67%) as litter to

represent organic matter. The artificial soil had no invertebrates, which play a key role in processing detritus and infuse the soil with physically smaller and chemically decomposed resources. We therefore mill grinded the litter (Retsch MM 400; 30 Hz for 30 s) and sieved it to < 250µm. Subsequently, the litter was mixed thoroughly with the artificial soil. Following the method by Caesar-TonThat (2002). The mixture was thoroughly wetted with ultrapure water, dried at 60°C for 48h and the resulting soil cake was ground and sieved through 2 mm sieves; i.e. the artificial soil comprised primary particles, litter fragments not exceeding 0.25mm, and artificial aggregates of various sizes not exceeding 2mm. Subsequently, this mixture was autoclaved twice on two consecutive days.

4.3.3 Soil microbial inoculation (different diversity levels)

The soil inoculum was obtained from a previous experiment from our lab (Yang et al., 2020). Soil was collected from an experimental field of Freie Universitaet Berlin (Berlin, Germany,) characterized as a sandy loam soil texture (for more soil properties see Leifheit et al. (2015)), and used as soil inoculum. 6 serial dilution steps (through 10^{-6}) were performed in autoclaved soil, each of these different dilutions were subsequently used as inoculum for the next dilution step (Franklin et al., 2001). Soil inoculums were incubated at room temperature for 2 months to achieve equivalent microbial biomass before use. 10^{0}(undiluted), 10^{3}diluted and 10^{6}diluted soil inoculums were used in our experiment. We air dried the inoculum soil in a sterile hood and sieved it through a 250µm mesh. We then added 1g of dry soil inoculum to 30g of sterile artificial soil, mixed carefully until the inoculum was homogeneously distributed in the artificial soil, to establish the respective diversity levels.

4.3.4 Incubation

We used 50ml centrifugation tubes containing the 30 (+1) g of soil, with hydrophobic vented caps to allow gas exchange. The water content was adjusted to 60% water holding capacity. We replenished water loss in a sterile hood by injecting water with a syringe twice a week. The incubation lasted 75 days.

4.3.5 Temperature treatment

The ambient temperature was set at 25°C. We started the experiment with all treatments at 25°C and incubated the tubes for 30 days, to allow full recovery of the microbial communities. After these 30 days the treatment with the elevated temperature was applied to half of the tubes.

This was done by increasing the temperature by 2.5℃ from 25 to 27.5℃ and keeping the temperature constant at 27.5 °C for the remaining duration of the experiment, which was 45 days. Total incubation time was 75 days (30+45 days). The 10 replicates of one factor combination were split into 3 groups and incubated in 3 individual incubators (temperature treatment: n=3).

4.3.6 Harvest/destructive sampling and Sample preparation

Soil samples were homogenized by spatula in a plastic bag, then dried at 40℃ for 48h in a drying oven and subsequently stored at room temperature until the analysis of water stable aggregates and total C (TC).

4.3.7 Respiration and C loss

We measured soil respiration as CO_2 production rate (ppm h^{-1}) on day 75 of the incubation. Before the measurement, we flushed each of the tubes with CO_2-free air for five minutes to standardize among experimental units (Rillig et al., 2019). After 5.5 hours (preliminary measurements showed that after this time readouts were within the range of the calibration curve), we sampled 1 ml of air from the headspace of each tube and subsequently injected this sample into an infrared gas analyser (LiCOR 6400xt).

The total C loss was calculated by the difference in total C (TC) before and after the incubation of 75 days.

4.3.8 Water-stability of soil aggregates (WSA)

We followed the protocol by Kemper and Rosenau (2018): the dried soil was sieved through a 2 mm sieve and we placed 4.0 g of soil into sieves for capillary rewetting in deionized water for 5 min. We used 250 μm sieves to test the stability of the soil fraction > 250 μm (macroaggregates) against water as disintegrating force. For the test, sieves carrying the wetted soil samples were placed in a wet-sieving machine (Eijkelkamp, Netherlands) and moved vertically (stroke = 1.3 cm, 34 times min^{-1}) for 3 min. The fraction (< 250 μm) left in the metal bin was transferred to a 53 μm sieve to repeat the wet-sieving again in order to obtain microaggregates. The fractions left on the sieves were dried at 60 ℃ for 24 h. There was no coarse matter (the sizes of sand and original organic matter fraction were smaller than 250 μm) in macroaggregates. The calculation of percent water stable aggregates was: %WSA= water stable aggregates (g) / 4.0 g. We stored macroaggregates and microaggregates at room

temperature until the analysis of SOC in different soil fractions. The fraction <53 μm, considered as free minerals consisting of free silt and clay, was not collected in our study.

4.3.9 Soil organic carbon fractions

For this study, we divided SOC fractions into three groups: 1. **free light fraction SOC (fLF-C)**; 2. **aggregate-associated C,** which consists of macro- and microaggregate-associated C; 3. **free mineral-associated C**.

We wet-sieved the soil with a 250 μm sieve and collected the material that passed through the sieve for the determination of microaggregates and fLF. The material that stayed on the sieve was collected for the determination of macroaggregate-associated C.

We followed and modified a protocol by Plaza et al. (2012) to separate microaggregates and fLF: To separate free microaggregates and fLF, the microaggregates of two replicates were combined due to the limited amount of microaggregates in each replicate, and transferred to a 15ml tube. We performed a density separation of soil organic carbon with the following steps: i) we added 12ml of sodium polytungstate (SPT) solution (Smetu, Germany) at a density of 1.85g ml^{-1} to the sample, ii) rotated the tube at 1 revolution/s for 30s, iii) centrifuged it at 2500g for 30min, iv) vacuum-filtered the supernatant (47mm, Glass microfiber filters - GF/A Grade 1.6μm pore size, Whatman, UK) and finally v) rinsed the material retained on the filter (free particulate OM) with 300ml of distilled water. Steps i) to v) of this density separation were repeated in order to more completely capture fLF. The remaining soil in the tube was designated as the microaggregates. To clean the SPT, this fraction was transferred into a 50ml tube, suspended in 30ml distilled water and centrifuged at 3000 g for 5 min; the supernatant was siphoned off and these steps were repeated three times.

All the fractions were dried at 60 °C for 24 h, then were ball milled (Retsch MM 400; 30 Hz for 3 min) for CN analysis using an Elemental Analyzer (EuroEA, HekaTech, Germany), the macroaggregates of two replicates were also combined for C measurement, the free mineral-associated C was calculated by subtracting macroaggregate-associated C and fLF-C from TC.

4.3.10 Statistical and data analysis

We analysed the effects of microbial diversity and elevated temperature by using two-way ANOVAs. We used a Shapiro Wilk's test (Royston, 1995) and Bartlett test (Boos, 2014) to check the normality of residuals and the homogeneity of variances, respectively, with a P-value cutoff of 0.05. We applied generalized least square models in the "nlme" package when conditions for homoscedasticity were not met (Pinheiro et al., 2020). All statistics were conducted in R 3.5.3 (R Core Team, 2017) with the basic packages, while the plots were created with the graphic package 'ggplot2' (Hadley and Winston, 2016).

4.4 Results

4.4.1 Free light fraction SOC (fLF-C), C loss and respiration rate

Microbial diversity had significant effects on both fLF-C and C loss ($p < 0.05$): low diversity had lower fLF-C and higher C loss than medium and high diversity (Fig. 4.2). However, temperature had no significant effects on fLF and C loss ($p = 0.07$ and $p = 0.91$, respectively), except soil with medium diversity had higher fLF-C at elevated temperature. The fLF-C in soil showed a negative correlation with C loss ($r = -0.49$, $p < 0.05$). Both microbial diversity and temperature had significant effects on the respiration rate ($p = 0.003$ and $p = 0.03$, respectively): low diversity had higher respiration than medium and high diversity, elevated temperature increased respiration rate.

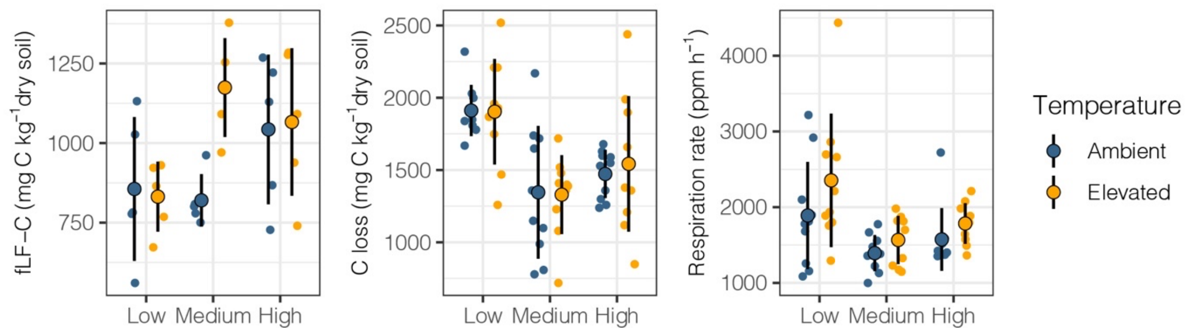

Figure 4.2 Effects of microbial diversity and temperature on fLF-C (a), C loss (b) and respiration rate (c). The points show the raw data of fLF-C (n=5), C loss (n=10) and respiration rate (n=10) with corresponding mean and standard deviation.

4.4.2 Water-stability of soil aggregates

The temperature and microbial diversity had significant interactive effects on the percentages of macroaggregates and microaggregates (Table 4.1). The percentage of macroaggregates decreased with increasing levels of microbial diversity, while at medium diversity macroaggregates were still high at ambient temperature (Fig. 4.3a). The percentage of macroaggregates in soil with medium diversity at ambient temperature was on average 19.59% higher than at elevated temperature (Fig. 4.3b). The percentage of microaggregates was negatively correlated with the percentage of macroaggregates (r= -0.97, $p < 0.0001$), showing the opposite pattern compared to macroaggregates.

Only temperature had significant effects on the percentage of total aggregates and free minerals ($p = 0.02$ and $p = 0.02$, respectively), the soil contains a higher percentage of aggregates and a accordingly lower percentage of free minerals at ambient temperature than at elevated temperature (Fig. 4.3c, d), as a trade-off between aggregates and free minerals.

Table 4.1 ANOVA results for the effects of temperature, levels of microbial diversity and the interaction of these factors. We applied linear model with function "lm" and generalized linear model with function "gls" (Tobergte and Curtis, 2013) whenever appropriate.

Source	F value	P value	F value	P value	F value	P value	F value	P value
	fLF		C loss		Respiration rate		Percentage of macroaggregates	
Temperature	3.30	0.08	0.030	0.86	**4.83**	**0.03***	**5.18**	**0.03***
Microbial diversity	**3.55**	**0.04***	**14.73**	**7.85e-06 *****	**6.60**	**3e-03****	**14.67**	**8.16e-06 *****
Temperature × Microbial diversity	2.97	0.07	0.10	0.91	0.29	0.75	**5.49**	**6.78e-03****
	The percentage of microaggregates		Percentage of aggregates		The percentage of minerals		Content of macroaggregates-associated C	

Temperature	**7.96**	**6.70e-03****	**5.67**	**0.02***	**5.67**	**0.02***	**4.83**	**0.03***
Microbial diversity	**11.39**	**1e-04*****	2.07	0.14	2.07	0.13	**6.60**	**3e-03****
Temperature × Microbial diversity	**7.01**	**2e-03****	1.64	0.20	1.64	0.20	0.29	0.75
	Content of microaggregates-associated C		Content of aggregates-associated C		Content of free minerals-associated C		Concentration of macroaggregates-associated C	
Temperature	0.28	0.60	**16.04**	**5e-04*****	**9.59**	**4.92e-03****	3.48	0.07
Microbial diversity	3.21	0.06	2.32	0.12	1.60	0.22	2.30	0.12
Temperature × Microbial diversity	**3.62**	**0.04***	1.47	0.25	0.27	0.77	0.05	0.95
	Concentration of microaggregates-associated C		Concentration of aggregates-associated C		Concentration of free minerals-associated C			
Temperature	4.24	0.05	**13.56**	**1.17e-03****	0.04	0.85		
Microbial diversity	0.45	0.64	2.55	0.10	0.89	0.42		
Temperature × Microbial diversity	0.53	0.59	1.72	0.20	0.81	0.46		

The asterisks, *, **, *** denote significant differences ($p < 0.05$, < 0.01 and < 0.001, respectively)

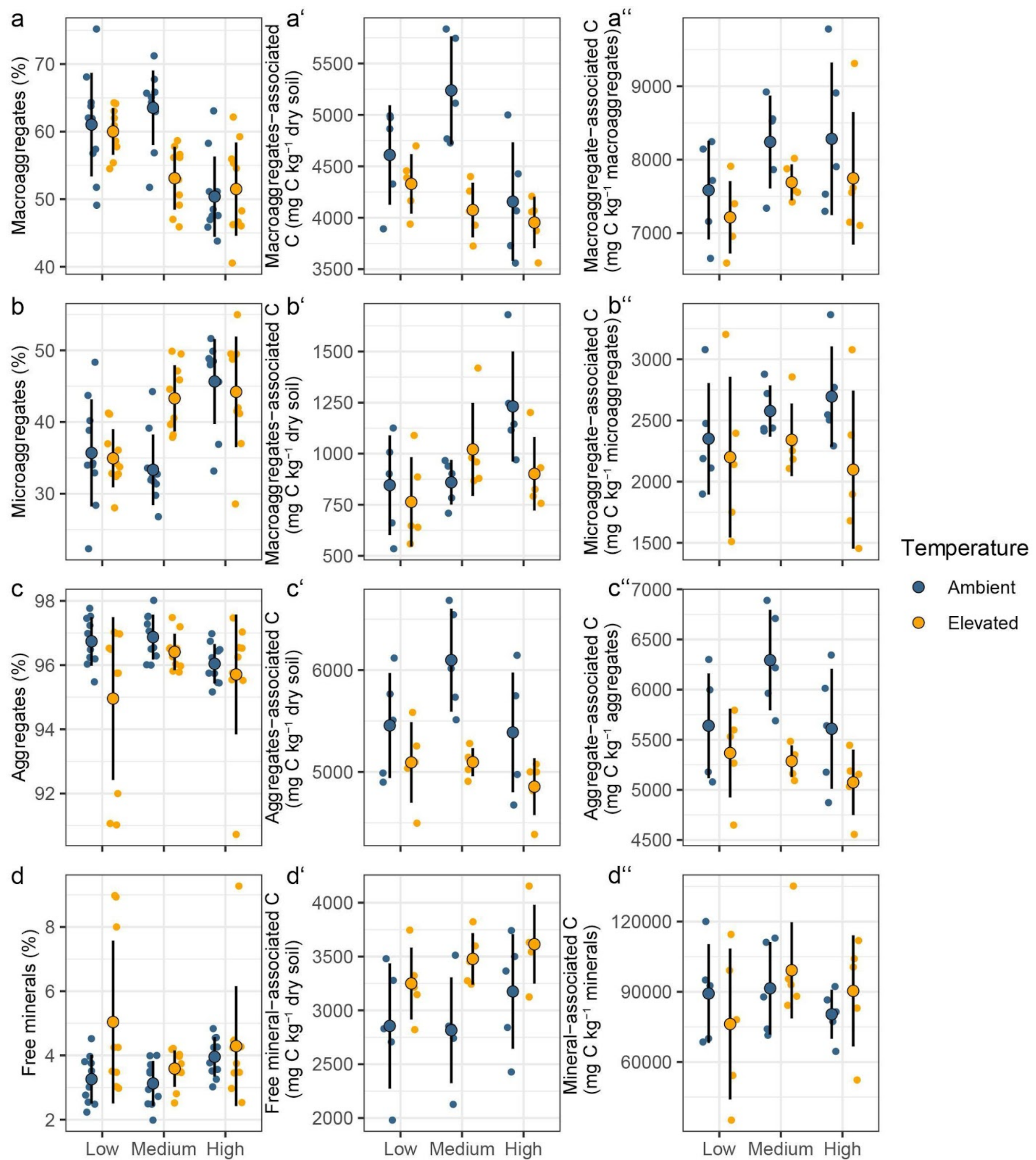

Figure 4.3 Effects of microbial diversity and temperature on the percentages of soil fractions: macroaggregates (a), microaggregates (b), aggregates (combination of macro- and microaggregates) (c) and free minerals (d); on fraction-associated C contents in soils (mg C kg^{-1} soil) and C concentrations in fractions (mg C kg^{-1} fraction): macroaggregate-associated C (a, a'), microaggregate-associated C (b, b'), aggregate-associated C (c, c') and free mineral-associated C (d, d'). The points show the raw data (n=5) with corresponding mean and standard deviation.

4.4.3 Aggregate-associated C

Macroaggregate-associated C

Both temperature and microbial diversity had significant effects on the content of macroaggregate-associated C: the content of macroaggregate-associated C decreased with increasing levels of microbial diversity, except medium diversity at ambient temperature, and ambient temperature had a higher content of macroaggregate-associated C (Fig. 4.3a'). The content of macroaggregate-associated C was positively associated with the percentage of macroaggregates (r= 0.74, p < 0.0001).

There were no statistical effects of elevated temperature on the C concentration in macroaggregates, while we observed macroaggregates at ambient temperature had relatively higher C concentration than at elevated temperature (Fig. 4.3a'').

Microaggregate-associated C

Temperature and microbial diversity had significant interactive effects (p = 0.04) on the content of microaggregate-associated C (Fig. 4.3b'), which was positively associated with the percentage of microaggregate (r= 0.51, p = 0.004), showing the similar pattern as the percentage of microaggregates.

There were no statistical effects of microbial diversity and temperature on the C concentration in microaggregates, however, we still observed microaggregates at ambient temperature had a relatively higher C concentration than at elevated temperature (Fig. 4.3b'').

Total aggregate-associated C

Temperature had significant effects on both the content of aggregate-associated C and the C concentration in aggregates (p = 5e-04 and p = 1.17e-03, respectively), which were higher at ambient temperature than elevated temperature regardless of microbial diversity levels (Fig. 4.3c', 4.3c'').

Mineral-associated C

Elevated temperature significantly elevated the content of free mineral-associated C (Fig. 4.3d'), while there were no significant effects of temperature and microbial diversity on C concentration in free minerals (Fig. 4.3d'').

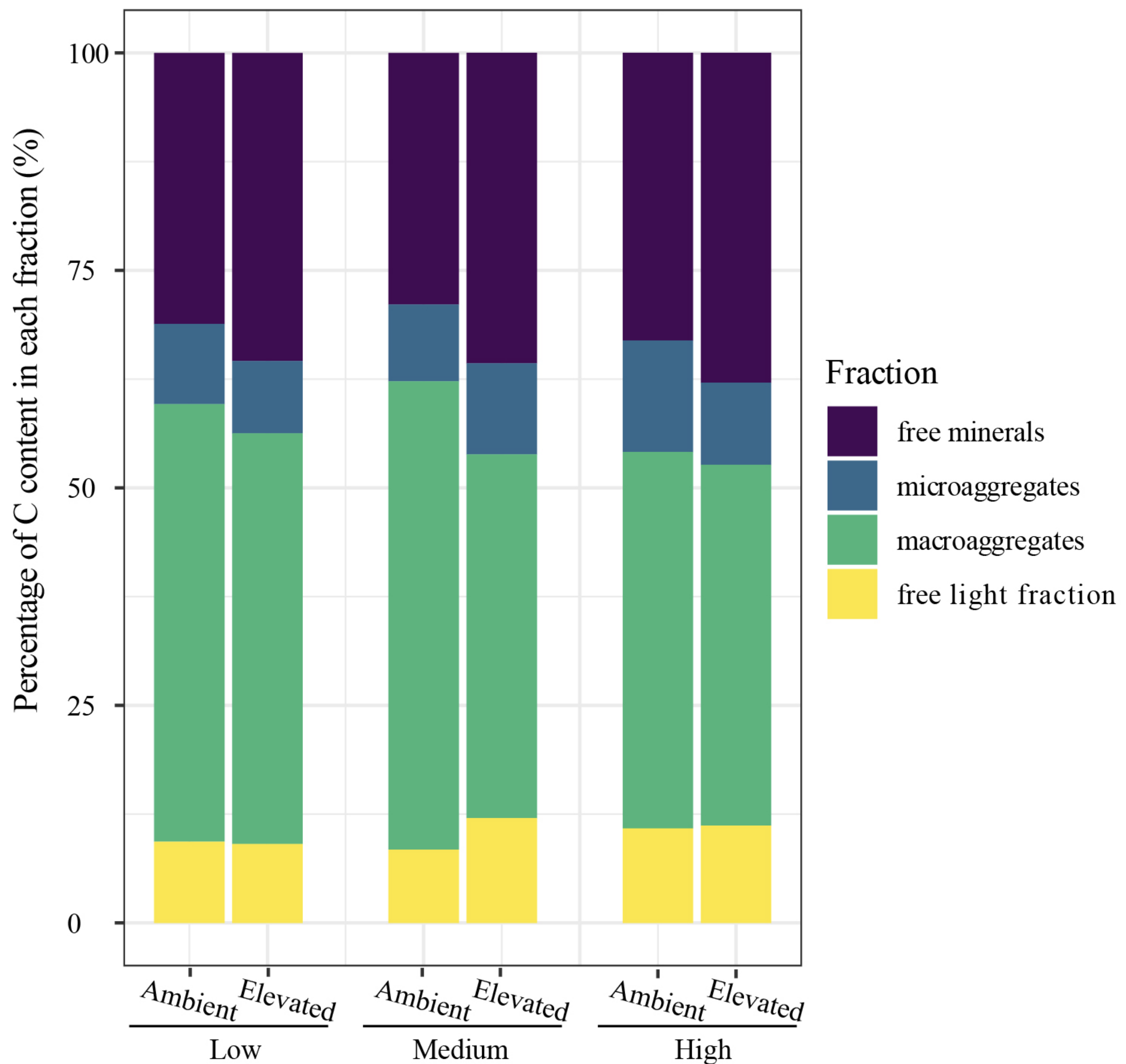

Figure 4.4 Effects of microbial diversity and temperature on SOC distribution in different soil fractions: fLF, macroaggregate, microaggregate and minerals. Values shown are means of five replicates.

4.5 Discussion

Our study findings support our hypothesis that elevated temperature will lead to greater decomposition of litter, reflected by CO_2 emission (Fig. 4.2c), but decrease the formation of aggregate-protected SOC, shown by the decrease in the content of aggregate-associated C and the concentration of C in aggregates. However, we observed an enhanced decomposition, revealed by increases in C loss, and more macroaggregates in soil with low diversity, which

are opposite to our hypothesis; nevertheless, the formation of aggregate-protected SOC was not affected by diversity levels.

4.5.1 Aggregates

Elevated temperature decreased the formation of total aggregates. One possible reason could be that higher temperature accelerated the decomposition of binding agents (Sollins et al., 1996). The other possible reason could be that microorganisms involved in aggregation might be sensitive to the heat stress and were suppressed (Rillig et al., 2002), resulting in less total aggregates at elevated temperature than ambient temperature. Ambient temperature especially had a much higher amount of macroaggregates than elevated temperature in soil with medium microbial diversity. The medium diversity level probably included highly efficient aggregators, causing substantial increased soil stability at ambient temperature.

Contrary to our expectations, the amount of macroaggregates decreased with increasing levels of microbial diversity. The dilution of microbial diversity/richness in the "low diversity" level might have selected for microbes that are better aggregators, such as saprophytic fungi (Lehmann and Rillig, 2015). Good soil aggregators such as fungi contribute to the stabilization of soil particles by entanglement of particles with hyphae or by exuding gluing substances (microbial exudates such as polysaccharides). Moreover, decomposition was faster in soil with low diversity as indicated by higher C loss, which provided microbes with energy and nutrient resources for active metabolism and exudation and the degraded organic matter could be bonded to clay particles (forming stable organo-mineral-complexes) (Oades, 1967, 1988). We assume that the overall stimulation of microbes at the low diversity level caused an increased production of glues, which are transient or temporary binding agents that might have contributed to the stabilization of soil aggregates in our short-term incubation (Tisdall and Oades, 1982; Pronk et al., 2012; Costa et al., 2018).

Soil with medium diversity had a similar amount of water stable macroaggregates compared to the low diversity treatment but reserved more SOC. Some microbial communities are more likely to produce glue-like agents under a certain temperature, whilst mineralization of organic matter is kept low (Gao et al., 2013). Thus, the microbial community in soil with medium diversity at ambient temperature could facilitate macroaggregate formation but in parallel was not actively decomposing OM and lost less carbon compared to the low diversity. This could explain the contradictory results of the relationship between microbial

diversity and their function of decomposition: the function might not have a linear relationship with microbial diversity (Pennekamp et al., 2018).

4.5.2 Aggregate-protected SOC

Temperature changed the distribution of SOC between aggregates and free minerals, that ambient temperature led to more SOC allocated in aggregates. One reason for this distribution was a higher percentage of aggregates at ambient temperature brought about the accumulation of aggregate-associated C, especially the higher percentage of macroaggregates at ambient temperature. The aggregate-associated C was mainly contributed by macroaggregate-associated C (Fig.3) due to the considerable proportion of macroaggregates in soil. The other reason explaining the distribution was a higher C concentration in aggregates at ambient temperature. Both microaggregates and macroaggregates had higher C concentrations at ambient temperature. This was probably due to elevated temperature had accelerated decomposition of SOC in aggregates revealed by the respiration. It might also be attributed to that microorganisms produced more microbial-derived C at ambient temperature, indicating higher carbon use efficiency (CUE). CUE is another parameter influencing the carbon storage in soil (Dignac et al., 2017). It has been reported that CUE can be higher at ambient temperature than at elevated temperature (Manzoni et al., 2012; Bölscher et al., 2017; Li et al., 2019), which has led to a greater immobilization of C in microbial-derived carbon (Kallenbach et al., 2016). Additionally, ambient temperature might have slower turnover of microbial-derived carbon (Li et al., 2019). Therefore, more C was preserved in aggregates at ambient temperature, which supported our hypothesis that elevated temperature attenuated the formation of aggregate protected C.

The content of aggregate-associated C and the C concentration in aggregates were highest in soil with medium microbial diversity at ambient temperature compared to the other treatments. We suggest that the unique microbial community composition in this treatment facilitated macroaggregate formation and CUE, consequently led to the SOC stabilization through aggregate-protection. Therefore, we see the need of further research studying the influence of microbial communities and soil aggregation on SOC stabilization.

Overall, microbial diversity had no influence on aggregate-associated C, except for one treatment: the low microbial diversity increased the macroaggregate-associated C formation, which was contrary to our hypothesis. This was attributed to higher percentages of macroaggregates in soil with low diversity, however, the concentration of SOC in

macroaggregates was no longer higher in soil with low microbial diversity, as enhanced decomposition in soil with low diversity also led to more C loss.

4.5.3 Carbon loss and free light fraction (fLF)

Opposite to our hypothesis, low microbial diversity caused higher C loss compared to medium and high diversity, probably due to enhanced decomposition. fLF-C was the most vulnerable SOC fraction to decomposition, the degradation of which mainly contributed to total C loss (Zimmermann et al., 2012). At low diversity, fast growing decomposers or decomposers using easily available substrates might have outcompeted other groups. It is also possible that interspecies competition suppressed decomposition in the soil with medium and high diversity (Jayasinghe and Parkinson, 2008). However, we did not find a significant difference ($p > 0.05$) in C loss between medium and high diversity. The diversity effect on decomposition might only be significant at the species-poor end of the diversity gradient as shown in Mary's study (2004) and communities might have been at a better functional equilibrium at medium and high diversity. Elevated temperature tended to cause slightly higher respiration, but this effect was not reflected in the data of C loss. In numerous previous studies considerable C loss or augmented soil respiration at elevated temperature was shown (Crowther et al., 2016; Wang et al., 2016; Hicks Pries et al., 2017; Melillo et al., 2017; Bond-Lamberty et al., 2018). In our study the exposure time of the soil was relatively short and effects might be stronger after a longer exposure time (the elevated temperature treatment was only active for 45 days). Additionally, the temperature increase that we applied was comparatively small, only 2.5 °C, where while other studies used larger temperature differences (Geraldes et al., 2012; Wang et al., 2016; Hicks Pries et al., 2017; Melillo et al., 2017).

4.6 Conclusion

Both diversity and temperature played important roles in SOC protection, low diversity could transfer easily decomposable SOC into macroaggregate-associated C more quickly, while ambient temperature could reserve higher concentration of SOC in aggregates. Though low microbial diversity caused fast loss in fLF-C, the aggregate-protection of SOC was more influenced by temperature, showing that allocation of SOC within aggregates was sensitive to temperature. Therefore, elevated temperature could decrease soil carbon storage in the long term by attenuating the stabilization of SOC.

4.7 Author Contributions

YL designed, performed the research, and conceived and performed the data analyses. YL, EL, AL and MR wrote the manuscript and contributed to the final version of the manuscript.

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5. GENERAL DISCUSSION AND CONCLUSIONS

5.1 Discussion

The factors influencing aggregates were widely studied, however, the number of global change factors are increasing, and as a consequence the influences on aggregation in a future world are still unclear. This doctoral study is targeting warming, microplastic and microbial diversity loss, aiming to explore their effects on soil aggregation.

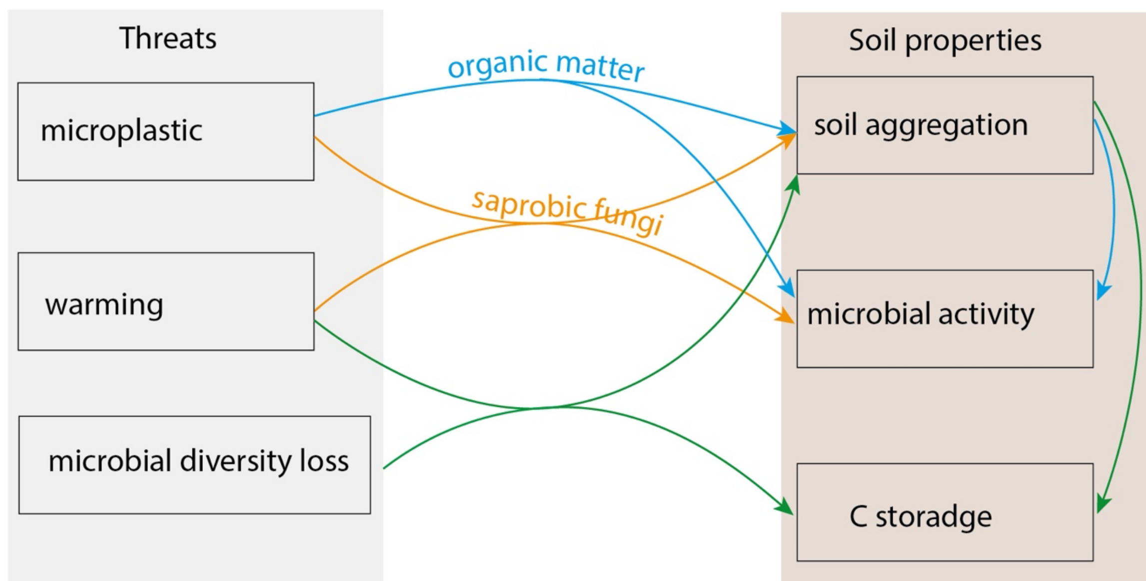

Figure 5.1 Conceptual overview of linkages between PhD works

The different colors represent three chapters (chapter1 = orange, chapter2 = blue, chapter3 = green). Arrows represent effects on soil properties.

Soil aggregation is essential to soil functions, maintains the microbial activity in soil and is involved in carbon protection. In chapter 2, we studied interactive effects of microplastic fiber and experimental warming on fungi-induced aggregation. We found that microplastic fibers only decreased the stability of aggregates in the presence of fungi, suggesting that the effects of microplastic fibers on soil aggregation were mediated by soil biota which are important for the formation of soil aggregates (Lehmann et al., 2019a). Elevated temperature could increase stable aggregates, the possible reason was both hyphal network and secretion of binding agents increased at higher temperature, leading to increased stable aggregates. However, microplastic fibers caused more negative effects on the stability of aggregates at elevated temperature. This phenomenon could be attributed to elevated temperature leading to a greater turnover of

macroaggregates, thus microfibers had more interactions with soil particles, likely to be entrapped within macroaggregates (Bläsing and Amelung, 2018; Zhang and Liu, 2018). Especially when higher temperature is favored by fungi, the formation of fungi-induced aggregates was enhanced, microplastic fibers might be incorporated into newly formed macroaggregates, leading to more macroaggregates with low stability.

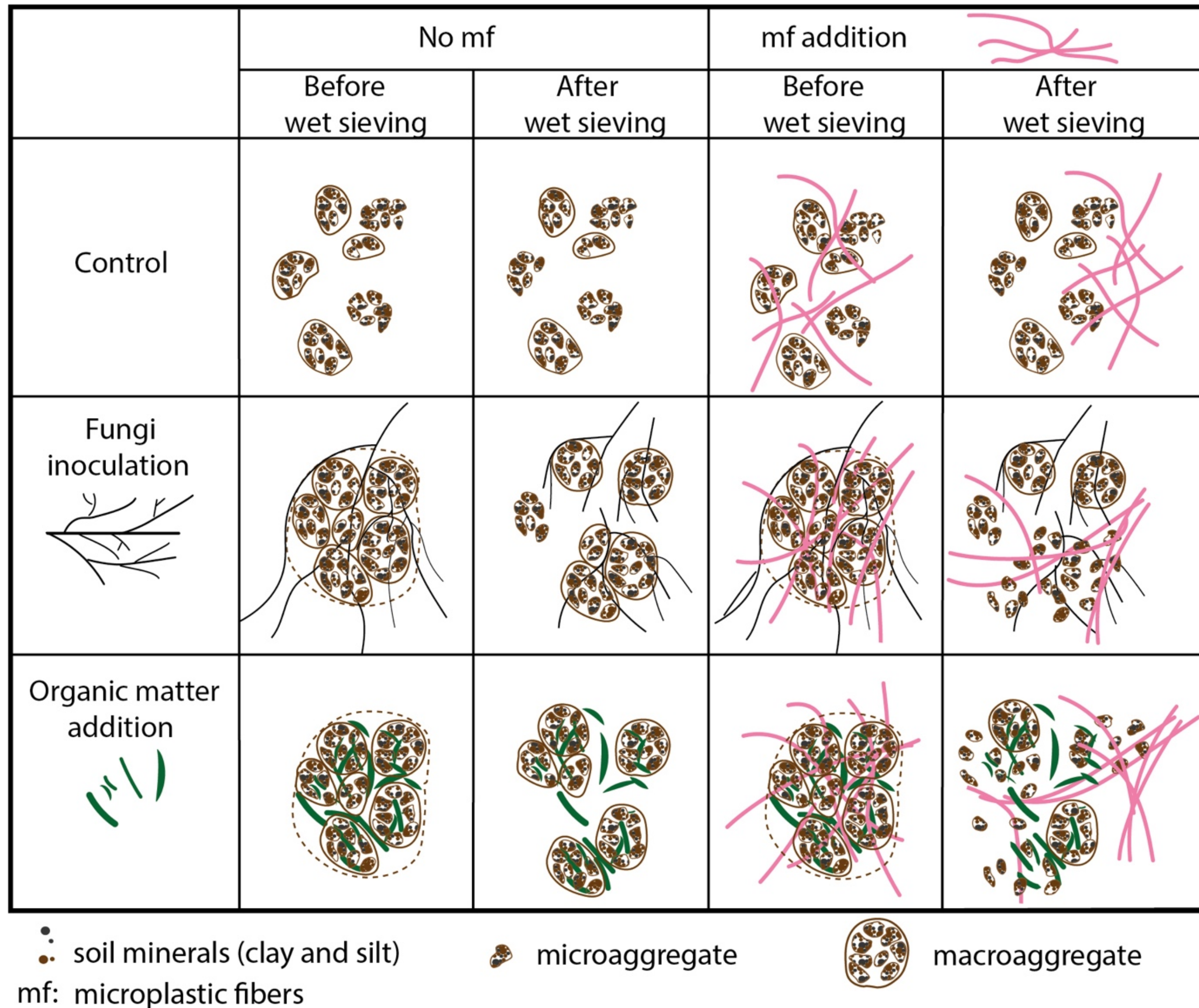

Figure 5.2 Conceptual overview of chapter 2 and chapter 3

From chapter 2, we found that effects of microplastic on soil aggregation depended on other soil conditions, we suggested that microplastic would have more negative effects when the conditions favored aggregate formation. To further test this hypothesis, we conducted the experiment of chapter 3. We found microplastic fibers had neutral effects on the stability of aggregates in soil without added organic matter but decreased the aggregate stability substantially in soil with added organic matter; the magnitude of the negative effects depended on the type of organic matter. Generally, the more beneficial the added organic matter was to the stability of aggregates, the more detrimental the microplastic fibers were to

the stability of aggregates. The possible reason was that organic matter addition stimulated microbial activity; therefore, the formation of aggregates was accelerated and the incorporation of fibers into newly formed aggregates increased as a consequence. This finding also holds true for the effects of microplastic fibers on soil enzyme activities: in chapter 3, microplastic fibers only had negative effects on enzyme activities in soil with organic matter addition; in chapter 2, microplastic fibers had more negative effects on enzyme activities at elevated temperature when elevated temperature was beneficial to enzyme activities. We assumed the incorporation of microplastic fibers into aggregates increased the opportunities for microfibers to interact with microbes, resulting in the decrease in enzyme activities. However, what caused this negative response in our study is unknown.

C storage is a pivotal soil function, which is sensitive to rising temperature (Crowther et al., 2016), and is mediated by microbial processes acting on soil organic matter (Allison et al., 2010). In chapter 3, we explored the interactive effects of experimental warming and microbial diversity loss on soil carbon protection by aggregates. We found both microbial diversity and temperature could influence the protection of soil organic carbon (SOC) by aggregates. Soil with low diversity had faster decomposition, leading to a higher formation of aggregates, thus more SOC are incorporated into macroaggregate. However, the concentration of SOC in macroaggregates was no longer higher in soil with low microbial diversity, as enhanced decomposition in soil with low diversity also led to more C loss. We found higher temperature led to less SOC allocated in aggregates. One reason was lower percentage of aggregates at higher temperature, especially the much lower percentage of macroaggregates at elevated temperature; the other reason was a lower C concentration in aggregates (both macroaggregates and microaggregates) at elevated temperature. The decreased concentration of SOC in aggregates at elevated temperature was attributed to the accelerated decomposition of soil organic matter at higher temperature. It might also have been due to microorganisms having lower CUE at elevated temperature (Manzoni et al., 2012; Bölscher et al., 2017). We found the highest percentage of aggregates formed in soil with intermediate microbial diversity at ambient temperature; we suggest some microbial communities are more likely to produce glue-like agents under a certain temperature (Gao et al., 2013), revealing the interactive effects of microbial community and temperature on the formation of stable aggregates.

5.2 Conclusions

Formation and stabilization of soil aggregates are dynamic and complex processes, mediated by many factors. Our soil is facing increasing threats due to climate change and anthropogenic activity. This doctoral work contributes to revealing the effects of elevated temperature, microplastics and microbial diversity loss on the stability of aggregates. The interactive effects of these factors might cause catastrophic decease in stable aggregates. We strongly suggest that global change effects should be analyzed not only as single factors but also in combination. In the end, further field and high-order interaction experiments are needed to test assertations in this doctoral study.

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APPENDIX A2: CHAPTER 2

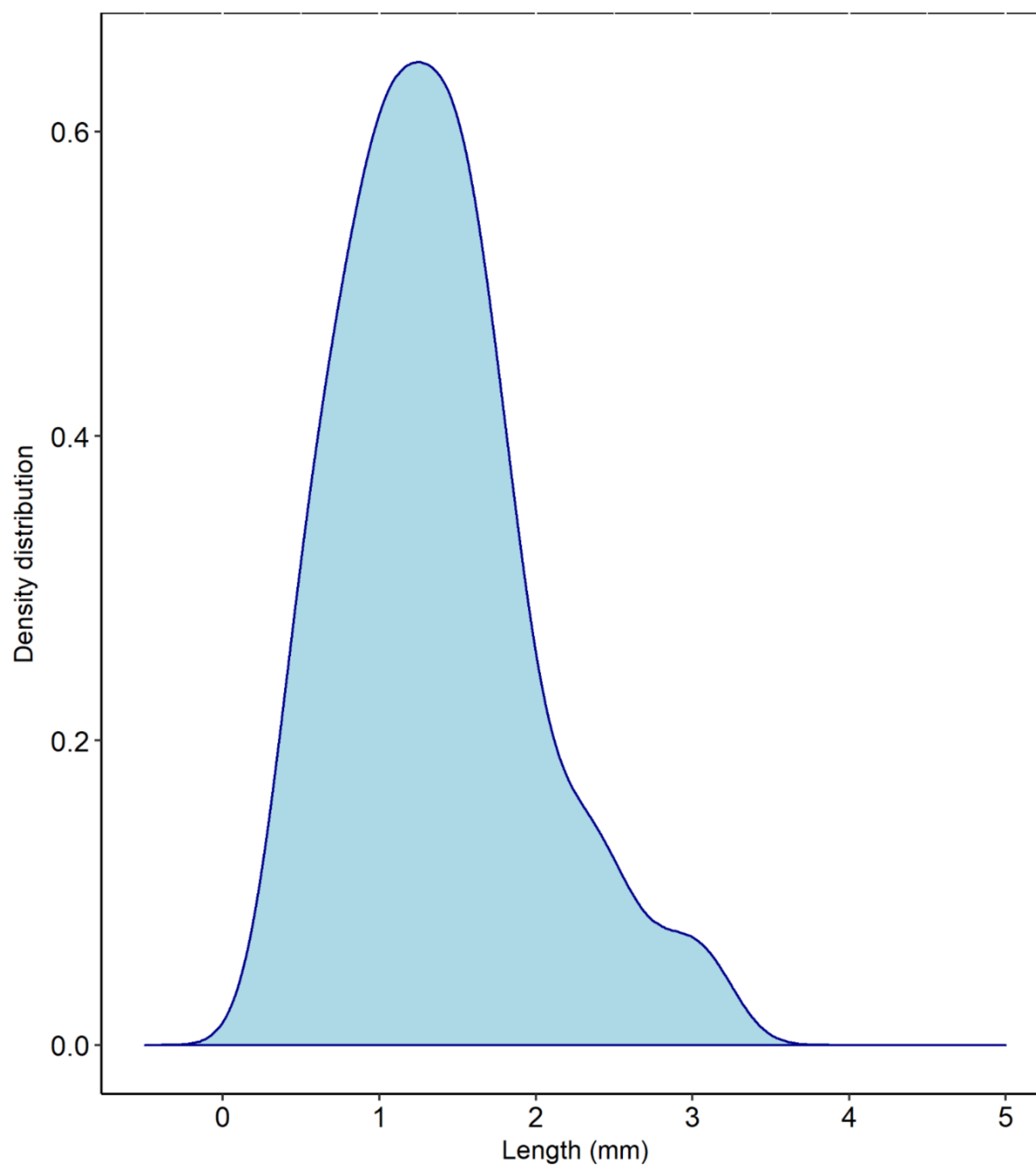

Figure A2.1. Microfiber size distribution. Polyacrylic fibers (N= 113) were measured under a microscope.

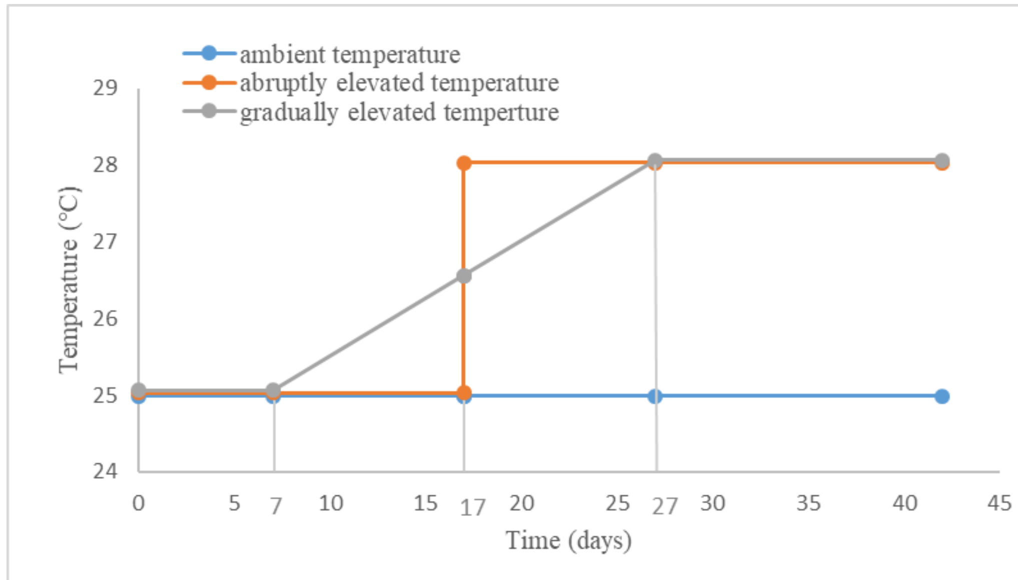

Figure A2.2. Depiction of temperature increase patterns. The ambient temperature was set at 25℃ while the elevated temperature was targeted at 28℃. For temperature elevation, two distinct increase patterns (abrupt and gradual) were explored. The two temperature elevation patterns started on different days (abrupt pattern: start on day 18; gradual pattern: temperature was increased by 0.15℃/day from day 8 to day 27), but both lasted until day 42 Following these two patterns, the average temperatures of abrupt and gradual increase are the same.

Table A.1. Information about phylum, order and Genbank and Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures GmbH, DSMZ) accession numbers of the five fungal strains used in this study.

Fungal strain ID	Taxonomic identification	Family	Order	Phylum	DSMZ	Radial colony growth rate (µm/h)
RLCS 01	*Mucor fragilis*	Mucoraceae	Mucorales	Mucoromycotina	DSM 100293	372.7625
RLCS 05	*Fusarium sp.*	Nectriaceae	Hypocreales	Ascomycota	DSM 100403	215.1519
RLCS 06	*Chaetomium angustispirale*	Chaetomiaceae	Sordariales	Ascomycota	DSM 100400	198.463
RLCS 07	*Amphisphaeriaceae strain 1*	Amphisphaeriaceae	Xylariales	Ascomycota	DSM 100284	196.82174
RLCS 08	*Gibberella tricincta*	Nectriaceae	Hypocreales	Ascomycota	DSM 100325	183.00533

Fungal isolates were identified based on the complete intergenic transcribed spacer (ITS) and a part of the large rRNA subunit (LSU). We used ITSx (Bengtsson-Palme et al., 2013) to split the rRNA sequences into the different subregions ITS1, 5.8S, ITS2 and LSU. Each region was aligned independently, using AlignSeqs in the R package DECIPHER 2.0 (Wright, 2015). Aligned subregion sequences were concatenated. Pairwise distances from sequences were calculated using the JC69 evolutionary model and used to construct a neighbor-joining tree, with the dist.ml and NJ functions respectively, of the R package "phangorn" 2.5.5 (Schliep, 2010). Root was placed at the midpoint of the longest path between any two tips. Taxonomic annotations of the fungal isolates were based on each subregions. We used the Naive Bayesian Classifier (Wang et al., 2007) as implemented in the R package "dada2" (Callahan et al., 2016) against UNITE database for ITS1 or ITS2 (Nilsson et al., 2018), and against the RDP LSU database for LSU (Cole et al., 2011). A confidence threshold was calculated with bootstrap analysis and an annotation was deemed valid when supported in 80% of the bootstraps. The best resolved taxonomic annotation among the regions was chosen. In the case of a conflict of taxonomic annotations between regions, priority was given to ITS1 or ITS2 because UNITE is more complete than the RDP LSU database. We followed the phylum classification by Spatafora et al. (Spatafora et al., 2017) (Table A2.1).

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Table A2.2. Plate counting of homogenates (replicates= 3, per fungal strain).

Fungal strain	Original numbers of fungal fragments	Numbers of fungal fragments after dilution
RLCS 01	ca.100	ca.100
RLCS 05	400, 500, 400	ca.40
RLCS 06	35, 44, 46	No dilution

RLCS 07	72,71,70	ca.70
RLCS 08	150, 145, 200	ca.75

Table A2.3. Percentage of water stable macroaggregates among different treatments.

Fungal species	Microfiber absent			Microfiber present		
	Ambient temperature	Gradually elevated temperature	Abruptly elevated temperature	Ambient temperature	Gradually elevated temperature	Abruptly elevated temperature
Control	15.46±6.90 Ca	18.93±10.49 Da	14.00±5.20 Da	22.36±6.87 Ba	20.55±8.09 Ba	19.71±8.14 Ca
RLCS 01	16.17±6.12 Cb	27.00±3.85 Ca	12.03±2.61 Db	30.13±7.74 Bab	34.84±9.42 Aa	23.81±10.74 Cb
RLCS 05	37.27±5.46 Ba	41.48±7.72 Ba	40.30±4.23 Ca	29.55±8.88 Ba	34.09±4.70 Aa	29.13±6.36 Ba
RLCS 06	50.63±6.36 Aa	36.91±3.78 Bb	50.38±6.11 ABa	25.78±5.89 Ba	27.43±5.50 Ba	16.98±3.90 Cb
RLCS 07	37.78±2.35 Bb	42.16±3.30 Bab	45.46±5.99 BCa	25.43±5.27 Ba	26.38±5.36 Ba	25.82±7.88 BCa
RLCS 08	49.15±5.55 Ab	57.55±2.59 Aa	52.71±6.28 Aab	44.43±8.17 Aa	36.84±7.24 Aa	42.75±7.46 Aa

Data are presented as mean and standard deviation. Different uppercase letters indicate significant differences (Duncan's test) among different fungi ($p < 0.05$). Different lowercase letters indicate significant differences (Duncan's test) among temperature treatments in terms of microfiber present or absent ($P < 0.05$).

APPENDIX A3: CHAPTER 3

respiration

We measured soil respiration as CO_2 production rate (ppm h^{-1}). Before the measurement, we flushed each of the tubes with CO2-free air for five minutes to standardize among experimental units (Rillig et al., 2019). After 2 hours (preliminary measurements showed that after this time readouts were within the range of the calibration curve), we sampled 1 ml of air from the headspace of each tube and injected this sample into an infrared gas analyser (LiCOR 6400xt).

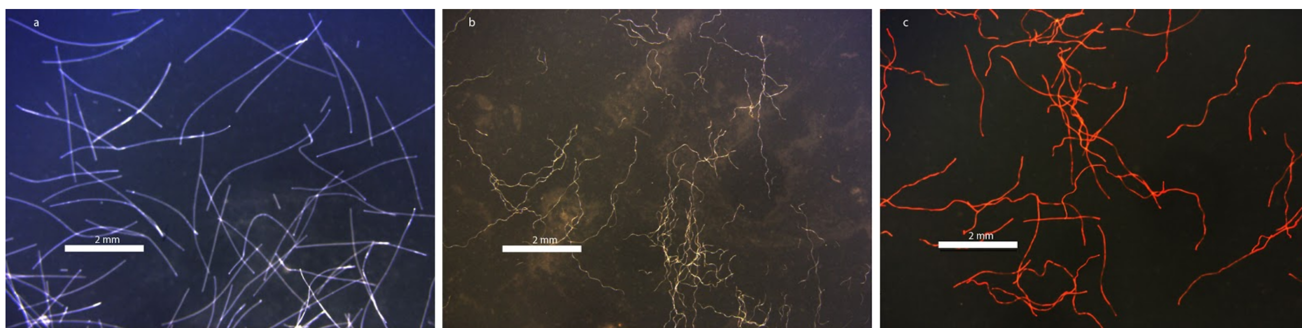

Figure A3.1. Three types of microplastic fibers used in our study: polyester1 (a), polyester2 (b), polyacrylic (c).

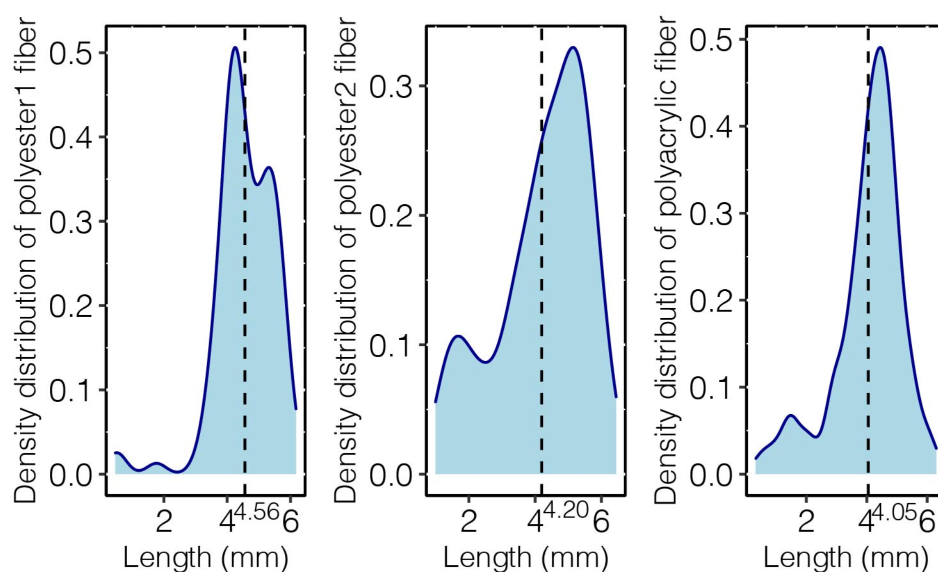

Figure A3.2. Size distributions of microplastic fibers (polyester1 (n = 102), polyester2 (n = 274), polyacrylic (n = 210)) used in our study. The dashed line indicates the mean value of fiber length.

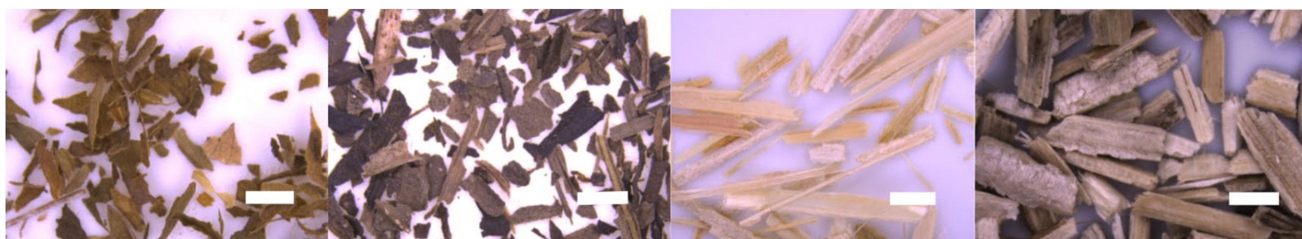

Figure A3.3. Four types of organic matters used in our study: *Medicago* leaves (a), *Plantago* leaves (b), wheat straw (c), hemp stem (d), the white bar represents 2mm size.

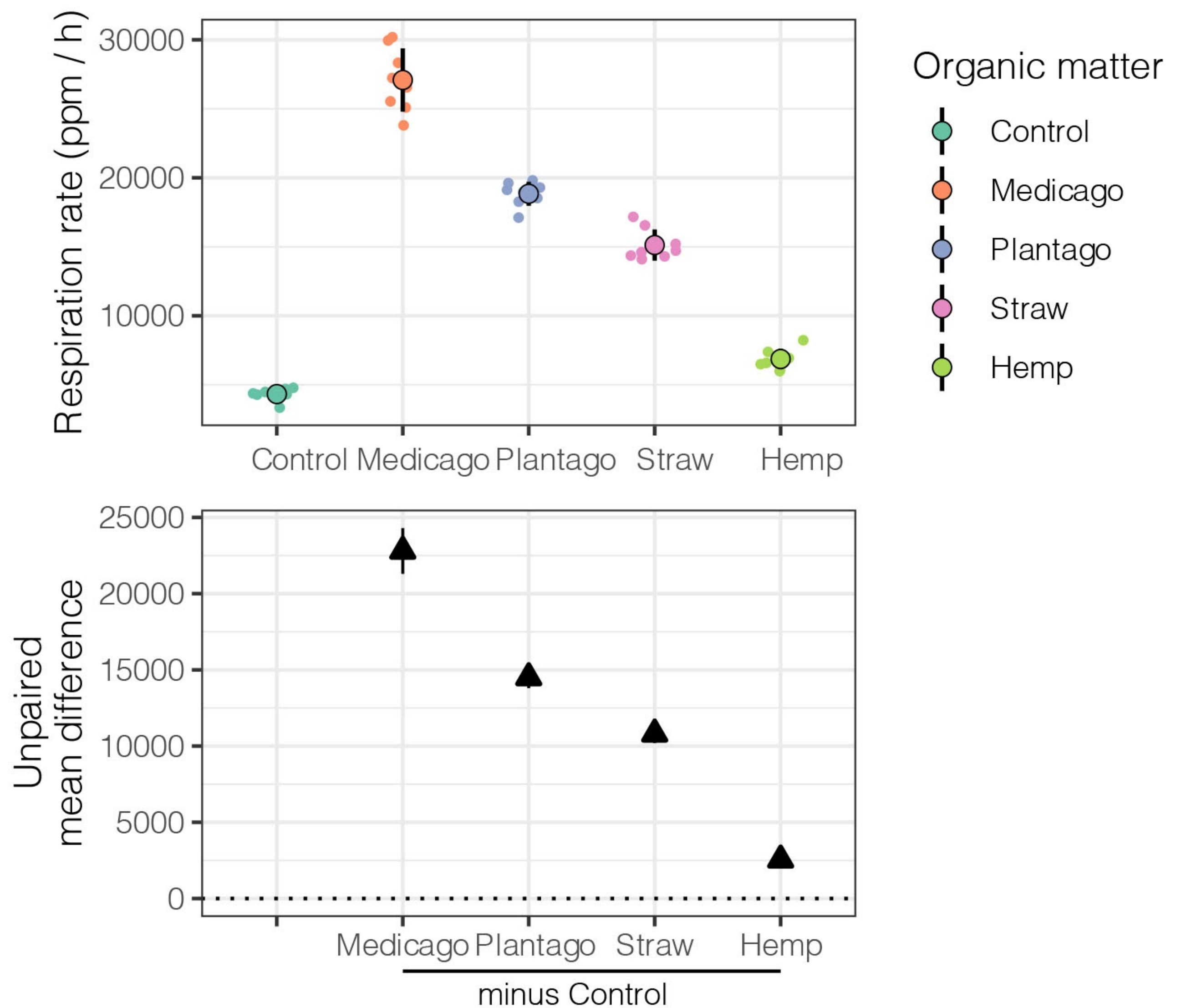

Figure A3.4. The soil respiration rate after the 1st day of incubation. Effects of organic matter on soil respiration rate. The upper panel shows the raw data of respiration, data distributions are aligned with corresponding mean and standard deviation (n = 8 for each treatment). The lower panel shows the unpaired mean differences of the organic matter addition and control. Triangles (arrow head up) represent the positive effect size mean (unpaired mean; effect magnitude) and the vertical lines the corresponding 95% confidence intervals (effect precision).

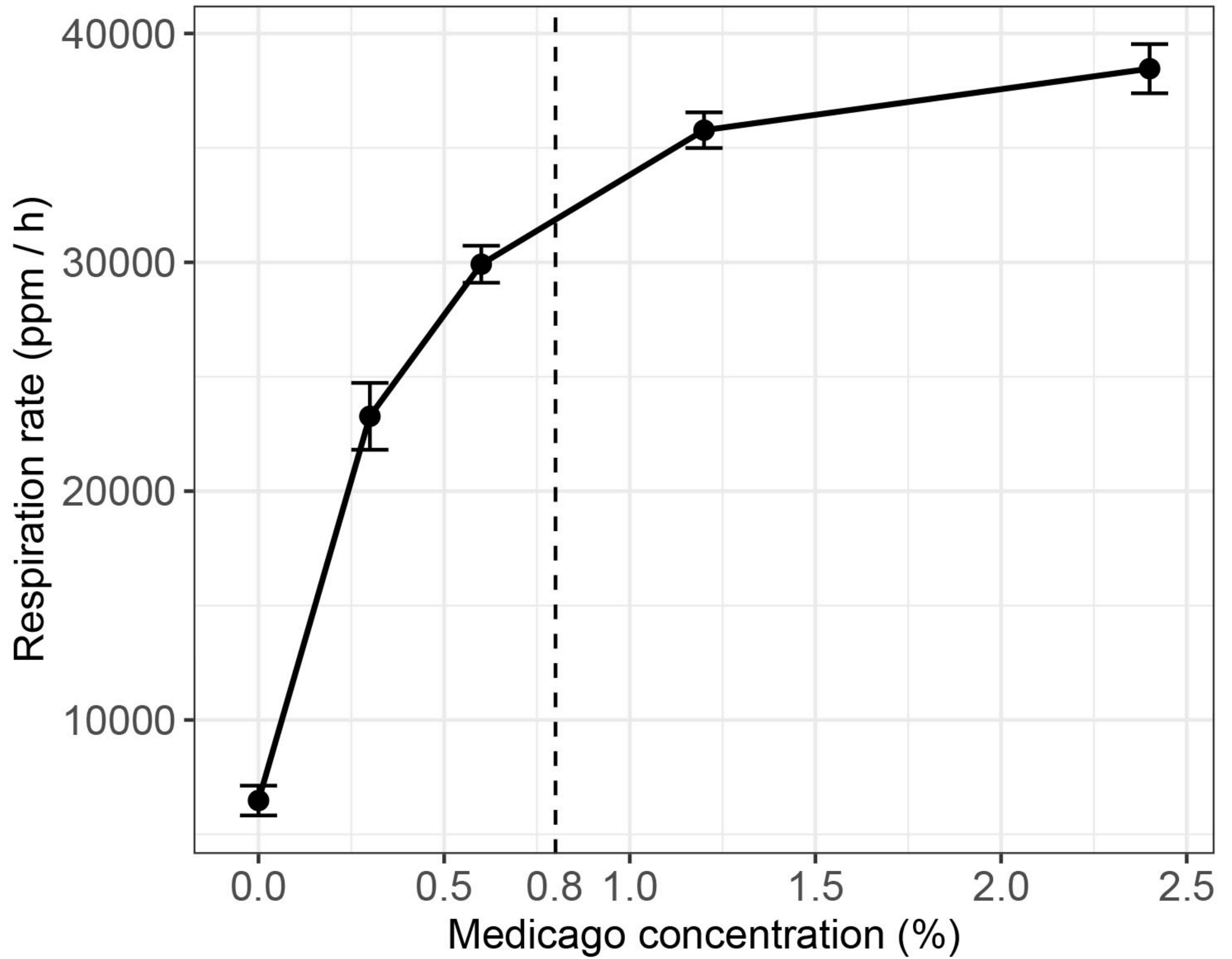

Figure A3.5. The respiration rates of soil added with different concentrations of *Medicago*. Data distributions are aligned with corresponding mean and standard deviation (n = 5 for each concentration). Given *Medicago* could contain the most content of labile substrates for microbes, we used a concentration below the saturation rate of *Medicago*. The saturation was determined as the soil respiration no longer increased with the increasing addition of *Medicago*.