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Microplastics interactions with soil organisms

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1 Summary

Microplastics (MP) are ubiquitous and likely represent a planetary boundary threat in terms of "chemical pollution and novel entities." MP are plastic particles between 100 nm – 5 mm of primary or secondary origin (intentionally produced or formed by abrasion processes during plastic use and environmental fragmentation). MP have different shapes (e.g., fibers, beads, fragments) and chemical composition (polymer type, additives, polymer mixtures). MP have proven to affect the biological fitness of aquatic life, enter the food web, and act as vectors of pollutants and pathogens in aquatic ecosystems. Evidence is emerging that agricultural soils are sinks for MP receiving MP through sewage sludges, plastic mulches, and organic fertilizers. MP are suspected threats to soil organisms and functions, but ecological consequences of MP in agricultural soils are widely unknown.

My thesis aimed to evaluate the risk of conventional and biodegradable MP compromising soil organisms and functions in agricultural soils. To assess the exposure risk of soil organisms towards MP, I considered background concentrations of MP and the fate of MP in agricultural soil. Given the critical role of soil microorganisms in maintaining C cycling, I studied the potential impacts of MP on soil microbial abundance, composition, and C turnover. Studies on the MP uptake and ecotoxicological consequences of MP on the biology of nematodes – key organisms of the soil food web – completed the risk assessment. I addressed these aspects by combining a microcosm study under controlled conditions with a field study and ecotoxicological tests with nematodes.

In the microcosm study, the influence of plastic type (conventional and biodegradable), particle size, and soil moisture on biodegradation of MP in soil and on effects on soil microorganisms were examined under controlled conditions (25 °C) for 230 days. After 104 and 230 days, the potential effects of MP on soil organisms and their processes were assessed based on soil microbiological indicators. These included phospholipid fatty acids (PLFAs) as biomarkers for the abundance and composition of the main soil microbial groups and activities of C cycling enzymes that drive the decomposition of differently complex substances as proxies for C turnover. A novelty of the microcosm study was the measurement of enzyme activities of individual MP particles, which were extracted from the soil after 230 days, which enabled us to understand better the role of MP as an interface for specific microbial processes, such as the enzymatic hydrolysis of MP, in the soil.

In the field study, MP background concentrations of the agricultural soil that did not receive treatment associated with MP entry (sewage sludges, organic fertilizers, plastic mulches) were

analyzed. In a randomized complete block design, the effects of MP, organic fertilizers (digestate and compost), and their interactions on soil microbiological indicators (microbial biomass, soil enzymes) were studied after 1, 5, and 17 months. The fate of added MP in the soil was evaluated by comparing MP particle-based concentrations in the soil after one month and 17 months and the concentrations at the start of the experiment.

In the nematode study, nematodes were exposed to MP feed suspensions on agar plates. The ingestion of MP particles through the ecotoxicological model nematode species *Caenorhabditis elegans* and the toxicity factors of plastic type and concentration on *C. elegans* were investigated in a combined reproduction and body length assay.

In all studies, artificially fragmented MP from a conventional polymer (low-density polyethylene, LDPE) and a biodegradable polymer blend (poly(lactic acid) and poly(butylene adipate-co-terephtalate), PLA/PBAT) were chosen as the model polymers. The presence of LDPE- and PLA/PBAT-MP in agricultural soils was considered realistic because of the application of these polymers as plastic mulches and compost bags. The soil used in my thesis, a silt-loam Luvisol, was from the site of the field study, which is a conventionally managed agricultural field of the research station Heidfeldhof, University of Hohenheim.

The topsoil of the field experiment contained 296 ± 110 (mean ± standard error) particles < 0.5 mm kg^{-1}, with polypropylene, polystyrene, and polyethylene dominating. Strikingly, the soil contained many red varnish particles > 0.5 mm. Both in the microcosm and field studies, LDPE- and PLA/PBAT-MP were persistent in the soil. In the microcosm study, a maximum of only 15% of PLA/PBAT-derived C was mineralized in relatively dry soil after 230 days (pF = 4, 25 °C).

Soil microbiological indicators did not suggest negative implications of MP on soil microorganisms and C turnover. However, at the microscale level, PLA/PBAT-MP exhibited cracks and enhanced lipase activities on their surface after 230 days of incubation in soil. Lipase activities on the surface of extracted PLA/PBAT were significantly higher than on LDPE particles and were associated with the highest mineralization degree (PLA/PBAT particles in dry soil). The surface-specific enzyme activities of MP were significantly higher than those of the bulk soil.

Nematodes ingested PLA/PBAT particles, evidenced by the presence of these particles in the digestive tract. MP reduced nematode reproduction by up to 23 % compared to the control group. This reduction was independent of plastic type and tended to be greater at higher concentration levels. There was no clear toxicity pattern of MP on nematode body length.

My thesis suggests that (1) agricultural soils, including those that do not face MP input through MP-containing sewage sludges, organic fertilizers, and through plastic mulches contain significant concentrations of various MP. This highlights the importance of diffuse inputs of MP to soils via atmospheric deposition or fragmentation of larger plastic fragments from improperly disposed of plastic waste (littering), but also through abrasion of machine coatings during tillage (a potentially newly identified MP input pathway), (2) also biodegradable MP can persist and show slow biodegradation in the soil. Thus, soil organisms will be exposed to conventional and biodegradable MP in the long term. With progressing biodegradation, biodegradable MP could become increasingly smaller with potentially higher risks for uptake by the soil fauna, (3) no acute negative consequences of MP for microorganisms and C turnover are expected. However, continuous MP inputs to agricultural soils, along with the persistence of MP, imply that MP will accumulate in soil; the risk of adverse interactions of MP with soil organisms will increase, (4) lipase activities on the MP-PLA/PBAT surfaces most likely contributed to surface erosion and depolymerization, paving the way for biodegradation of PLA/PBAT. These observations at the MP-soil interface suggest that MP form a microbial habitat - the plastisphere. How such plastisphere-specific processes affect soil C cycling and the microbial community in the long term cannot be predicted to date. The stimulation of microbial processes in the plastisphere could accelerate the decomposition of soil organic matter, thus reinforcing climate change effects due to faster C turnover in soils or leading to local nutrient depletion due to progressing biodegradation in the plastisphere, and (5) given the potential entry of MP into the soil food web via ingestion by nematodes, there could be ecological consequences as MP could be transferred from one trophic level to another, posing potential risks to members of higher trophic levels (e.g., earthworms). The potential reduction of nematode reproduction by MP could negatively impact soil functions, such as regulating biogeochemical cycles.

One should be aware that MP occur in agricultural soils along with other environmental stressors, such as chemical contamination and climate change. The interaction of MP with other disturbance factors was beyond the scope of my thesis. Clarifying these potential risks of interaction effects of MP and other environmental stressors on soil organisms and functions is imperative.

2 Zusammenfassung

Mikroplastik (MP) ist ubiquitär und stellt wahrscheinlich eine planetarische Grenzbedrohung im Zusammenhang von "chemical pollution and novel entities“ dar. Als MP werden Kunststoffpartikel zwischen 100 nm und 5 mm primären oder sekundären Ursprungs (absichtlich hergestellt oder durch Abriebprozesse bei der Verwendung von Kunststoffen und Fragmentierung in der Umwelt entstanden) bezeichnet. MP hat unterschiedliche Formen (z. B. Fasern, Perlen, Fragmente) und chemische Zusammensetzungen (Polymertyp, Zusatzstoffe, Polymermischungen). MP in aquatischen Ökosystemen kann nachweislich die biologische Fitness von Wasserlebewesen beeinträchtigen, in das Nahrungsnetz gelangen und als Vektor für Schadstoffe und Krankheitserreger fungieren. Landwirtschaftliche Böden sind vermutlich Senken für MP aufgrund von MP-Einträgen über Klärschlämme, Plastikmulche und organische Düngemittel. MP steht im Verdacht, Bodenorganismen und -funktionen zu gefährden, aber die ökologischen Folgen von MP in landwirtschaftlichen Böden sind weitgehend unbekannt.

Ziel meiner Doktorarbeit war es, das Risiko von konventionellem und biologisch abbaubarem MP hinsichtlich der Beeinträchtigung von Bodenorganismen und -funktionen in landwirtschaftlich genutzten Böden abzuschätzen. Um das Expositionsrisiko von Bodenorganismen gegenüber MP zu bewerten, berücksichtigte ich Hintergrundkonzentrationen von MP und den Verbleib von MP in landwirtschaftlichen Böden. Angesichts der entscheidenden Rolle der Bodenmikroorganismen bei der Aufrechterhaltung des Kohlenstoff(C) - Kreislaufs untersuchte ich die potenziellen Auswirkungen von MP auf die Abundanz, die Zusammensetzung und den C-Umsatz von Bodenmikroorganismen. Studien zur MP-Aufnahme und zu den ökotoxikologischen Folgen von MP auf die Biologie von Nematoden - Schlüsselorganismen des Nahrungsnetzes im Boden - vervollständigten die Risikobewertung. All diese Aspekte wurden anhand einer Mikrokosmosstudie unter kontrollierten Bedingungen, einer Feldstudie und ökotoxikologischen Tests mit Nematoden untersucht.

In der Mikrokosmenstudie wurde der Einfluss des Kunststofftyps (konventionell und biologisch abbaubar), der Partikelgröße und der Bodenfeuchtigkeit auf den biologischen Abbau von MP im Boden und auf die Auswirkungen von MP auf Bodenmikroorganismen unter kontrollierten Bedingungen (25 °C) über 230 Tage hinweg untersucht. Die möglichen Auswirkungen von MP auf Bodenorganismen und -prozesse wurden anhand von mikrobiologischen Indikatoren im Boden nach 104 und 230 Tagen bewertet. Die mikrobiologischen Indikatoren umfassten Phospholipidfettsäuren (PLFAs) als Biomarker für die Häufigkeit und Zusammensetzung der

wichtigsten mikrobiellen Gruppen im Boden und die Aktivitäten von Enzymen des C- Kreislaufs, die den Abbau unterschiedlich komplexer Substanzen steuern, als Indikatoren für den C-Umsatz. Ein Novum der Mikrokosmenstudie war die Messung der Enzymaktivitäten einzelner MP-Partikel, die nach 230 Tagen aus dem Boden extrahiert wurden. Dies ermöglichte ein besseres Verständnis der Rolle von MP als Schnittstelle für spezifische mikrobielle Prozesse, wie die enzymatische Hydrolyse von MP, im Boden.

In der Feldstudie wurden MP-Hintergrundkonzentrationen des landwirtschaftlich genutzten Bodens, der keine besondere Nutzungsgeschichte aufweist, die mit MP-Einträgen assoziiert ist (Klärschlämme, organische Düngemittel und Plastikmulche), analysiert. In einem randomisierten Blockdesign wurden die Auswirkungen von MP, organischen Düngemitteln (Gärreste und Kompost) und deren Wechselwirkungen auf mikrobiologische Indikatoren im Boden (mikrobielle Biomasse, Bodenenzyme) nach 1, 5 und 17 Monaten untersucht. Der Verbleib der zugesetzten MP im Boden wurde durch den Vergleich der Konzentrationen der MP-Partikel im Boden nach einem Monat und nach 17 Monaten mit den Konzentrationen zu Beginn des Experiments ausgewertet.

In allen Studien wurde künstlich hergestelltes MP aus einem herkömmlichen Polymer (Polyethylen niedriger Dichte, LDPE) und einer biologisch abbaubaren Polymermischung (Polymilchsäure und Poly(butylenadipat-co-terephtalat), PLA/PBAT) als Modellpolymere gewählt. Das Vorkommen von LDPE- und PLA/PBAT-MP in landwirtschaftlichen Böden wurde als realistisch angesehen, da diese Polymere für Kunststoffmulche und Kompostbeutel verwendet werden. Der für die Untersuchungen verwendete Boden, ein schluffig-lehmiger Luvisol, stammte vom Standort der Feldstudie, einer konventionell bewirtschafteten Ackerfläche der Forschungsstation Heidfeldhof der Universität Hohenheim.

Der Oberboden des Feldversuchs enthielt 296 ± 110 (Mittelwert ± Standardfehler) Partikel < 0,5 mm kg^{-1}, wobei Polypropylen, Polystyrol und Polyethylen dominierten. Auffallend war, dass in dem Boden viele rote Lackpartikel > 0,5 mm vorkamen. Sowohl in der Mikrokosmen- als auch in der Feldstudie waren LDPE- und PLA/PBAT-MP im Boden persistent. In der Mikrokosmenstudie wurden in relativ trockenem Boden nach 230 Tagen (pF = 4, 25 °C) maximal nur 15 % des PLA/PBAT-stämmigen C‘s mineralisiert.

Die mikrobiologischen Indikatoren ließen nicht auf negative Auswirkungen von MP auf die Bodenmikroorganismen und den C-Umsatz schließen. Auf mikroskaliger Ebene betrachtet, wiesen PLA/PBAT-MP jedoch Risse und erhöhte Lipaseaktivitäten auf ihrer Oberfläche nach 230 Tagen Inkubation im Boden auf. Die Lipaseaktivitäten auf der Oberfläche von extrahierten PLA/PBAT-Partikeln waren deutlich höher als auf LDPE-Partikeln und gingen mit dem

höchsten Mineralisierungsgrad einher (PLA/PBAT-Partikel im trockenen Boden). Die oberflächenspezifischen Enzymaktivitäten von MP waren signifikant höher als die des angrenzenden Bodens.

Nematoden nahmen PLA/PBAT-Partikel auf, nachgewiesen durch Partikel im Verdauungstrakt. MP reduzierte die Fortpflanzung der Nematoden um bis zu 23 % im Vergleich zur Kontrollgruppe. Diese Reduktion war unabhängig von der Art des Kunststoffs und fiel bei höheren Konzentrationen tendenziell stärker aus. Es gab kein klares Toxizitätsmuster von MP auf die Körperlänge der Nematoden.

Die Ergebnisse meiner Doktorarbeit legen nahe, dass (1) landwirtschaftliche Böden – auch solche, die nicht durch Klärschlämme, Plastikmulch und organische Düngemittel mit MP belastet sind – erhebliche Konzentrationen verschiedener MP enthalten. Dies unterstreicht die Bedeutung des diffusen Eintrags von MP in die Böden durch atmosphärische Ablagerung oder Fragmentierung größerer Kunststofffragmente aus unsachgemäß entsorgten Kunststoffabfällen („Littering“), aber auch durch Abrieb von Maschinenbeschichtungen bei der Bodenbearbeitung (ein möglicherweise neu identifizierter MP-Eintragspfad), (2) auch biologisch abbaubare MP im Boden verbleiben und nur langsam biologisch abgebaut werden. Daher werden Bodenorganismen langfristig konventionellen und biologisch abbaubaren MP ausgesetzt sein. Mit fortschreitendem biologischem Abbau könnten biologisch abbaubare MP zunehmend kleiner werden, was ein potenziell höheres Risiko für die Aufnahme durch die Bodenfauna darstellt, (3) keine akuten negativen Auswirkungen von MP auf Mikroorganismen und den C-Umsatz zu erwarten sind. Kontinuierliche MP-Einträge in landwirtschaftlich genutzte Böden sowie die Persistenz von MP bedeuten jedoch, dass sich MP im Boden anreichern werden; das Potenzial für negative Wechselwirkungen von MP mit Bodenorganismen wird zunehmen, (4) Lipase-Aktivitäten auf den MP-PLA/PBAT-Oberflächen höchstwahrscheinlich zur Oberflächenerosion und Depolymerisation beitrugen und den Weg für den biologischen Abbau von PLA/PBAT ebneten. Diese Beobachtungen an der MP-Boden-Grenzfläche legen nahe, dass MP einen mikrobiellen Lebensraum bilden – die Plastisphäre. Wie sich solche plastisphärenspezifischen Prozesse langfristig auf den C-Kreislauf im Boden und die mikrobielle Gemeinschaft auswirken, lässt sich bisher nicht vorhersagen. Die Stimulierung mikrobieller Prozesse in der Plastisphäre könnte den Abbau organischer Bodensubstanz beschleunigen und damit die Auswirkungen des Klimawandels aufgrund eines schnelleren C-Umsatzes in den Böden verstärken oder aufgrund des fortschreitenden biologischen Abbaus in der Plastisphäre zu einer lokalen Nährstoffverarmung führen, (5) angesichts des potenziellen Eintritts von MP in das Nahrungsnetz des Bodens über die Aufnahme durch Nematoden dies

ökologische Folgen haben könnte, da MP von einer trophischen Ebene auf eine andere übertragen werden könnte, was eine potenzielle Gefahr für Mitglieder höherer trophischer Ebenen (z. B. Regenwürmer) darstellt. Die potenzielle Verringerung der Reproduktion von Nematoden durch MP könnte sich negativ auf die Bodenfunktionen auswirken, z. B. auf die Regulierung biogeochemischer Kreisläufe.

Man sollte sich darüber im Klaren sein, dass MP in landwirtschaftlich genutzten Böden zusammen mit anderen Umweltstressoren, wie z. B. chemischer Kontamination und Klimawandel, auftreten. Die Wechselwirkung von MP mit anderen Störfaktoren lag außerhalb des Rahmens vorliegender Arbeit. Für die Klärung dieser potenziellen Risiken von Wechselwirkungen von MP mit anderen Umwelstressoren auf Bodenorganismen und -funktionen sind weitere Untersuchungen unerlässlich.

3 General introduction

Plastic pollution has been identified as one of the most severe global environmental problems and receives great attention in society, politics, and science (*Bank* and *Hansson*, 2019). The relevance of this topic manifests, e.g., in the "Directive of the European Union on the reduction of impacts of certain single-use plastics in the environment" (*European Parliament and Council*, 2019), which came into force in July 2021, and the "upcoming initiative on microplastics," which is part of the "European Green Deal"). Both aim to prevent and reduce plastic pollution in the environment, responding to concerns about the potential risks of plastics to ecosystems and human health.

The term plastics refers to a range of highly versatile synthetic polymers (*PlasticsEurope*, 2021), of which more than 5,300 are known today (*Jacquin* et al., 2019). With applications in packaging, building and construction, automotive, household, leisure and sports, and agriculture, plastics have revolutionized our daily life like no other material (*Rillig* et al., 2017a; *Agarwal*, 2020). Large-scale production and widespread use of plastics started in the 1950s, with an estimated global production of 2 Mt in 1950, peaking at 368 Mt in 2019 (a minimal decline by 1 Mt in 2020 was due to the COVID pandemics) (*PlasticsEurope*, 2021; *Geyer* et al., 2017). Plastics divide into thermoplastics and thermosets (*PlasticsEurope*, 2019). Thermoplastics can be repeatedly formed after heating and freezing, whereas thermosets cannot. The economically most important polymers are thermoplastics polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), and thermoset polyurethane (PUR) (*PlasticsEurope*, 2021).

Improper end-of-life management of plastics has led to a high accumulation of plastics in the environment. An estimated 60 % of the plastics manufactured between 1950 and 2015 have been discarded in landfills or emitted into the environment (amounting to approximately 4,900 Mt), and only around 9 % have been recycled (*Geyer* et al., 2017). The stable C backbones of most plastics render them recalcitrant to microbial degradation and are the reason they are likely to persist in the environment for decades, if not centuries (*Krueger* et al., 2015). This gigantic anthropogenic mismanagement is why plastics form their biogeochemical cycle in the environment involving "the continuous and complex movement of plastic materials between different abiotic and biotic ecosystem compartments, including humans (*Bank* and *Hansson*, 2019)."

Reports of plastic debris in the oceans date back to the 1970s (*Andrady*, 2011). Relatively late, minute plastic particles, called microplastics (MP), were recognized to accumulate in the oceans

(*Thompson* et al., 2004). *Rochman* et al. (2019) recently stated, "microplastics are not microplastics are not microplastics." This way, they expressed that MP are not a single compound but extremely diverse; they originate from numerous polymers, differ in color, size, and shape (e.g., fibers, fragments, films, spheres), and rarely occur alone but as a combination of different polymers and additives (*Rochman* et al., 2019). Primary MP are intentionally manufactured with abrasive functions in industry or contained in personal care products (e.g., microbeads as exfoliants in cosmetics). Secondary MP are generated during the utilization of a product (e.g., plastic microfibers from machine-washed clothing) or through environmental fragmentation by physical, biological, and chemical factors. Concerning their size, to date, there is no accepted definition range (*Hartmann* et al., 2019; *Rochman* et al., 2019), but mostly MP are defined as particles between 100 nm to 5 mm (*Souza Machado* et al., 2018; *Okoffo* et al., 2021; *Ng* et al., 2018). This is the definition that I considered in my thesis.

MP are of particular concern because there is extensive evidence for multiple adverse direct effects of MP on organisms in aquatic ecosystems (*Haegerbaeumer* et al., 2019; *Franzellitti* et al., 2019; *Foley* et al., 2018; *Eerkes-Medrano* et al., 2015; *Andrady*, 2017; *Galloway* et al., 2017; *Horton* et al., 2017). Aquatic organisms ingest MP. MP can reduce reproduction, growth, and fitness caused by internal damage such as lacerations and inflammations or lower food intake in aquatic organisms and has proven to accumulate in the food chain via trophic transfer. Moreover, MP are suspected of serving as a vector of harmful pathogen species and chemicals and as a habitat for aquatic microorganisms to elicit changes in biogeochemical cycles in the oceans (*Jacquin* et al., 2019; *Amaral-Zettler* et al., 2020). Due to these findings, plastic litter, i.e., weathering plastics, is currently being discussed to fulfill the criteria of being a planetary boundary threat within the context of "chemical pollution and the release of novel entities" (*Arp* et al., 2021). MP from weathering plastics already meet the first two criteria to be considered a planetary boundary threat, i.e., (1) exposure to the entire planet and (2) irreversibility of that exposure. If MP is also an ecological disruptor (3) is not yet clear (*Arp* et al., 2021).

Biodegradable plastics are promising alternatives to replacing conventional polymers (*Agarwal*, 2020; *Krueger* et al., 2015; *Folino* et al., 2020) because microorganisms can biodegrade them. Theoretically, biodegradable plastics leave no residue, thus ensuring an MP-free environment (*Agarwal*, 2020). Nevertheless, it is not yet clear if biodegradable plastics disappear entirely and, due to partial biodegradation, do not instead contribute to the in-situ formation of MP in the environment (*Napper* and *Thompson*, 2019; *Helmberger* et al., 2020; *Souza Machado* et al., 2018). With an estimated 1,6 Mt of nominally biodegradable plastics

produced in 2021, they have a relatively small share compared to conventional plastics (367 Mt in 2021; *European Bioplastics*, 2021). Nevertheless, biodegradable plastics are expected to more than triple by 2026. According to *European Bioplastics* (2021), the most used biodegradable plastics in 2021 were polylactic acid (PLA), polybutylene adipate co-terephthalate (PBAT), and starch blends. Polybutylene succinate (PBS) and polyhydroxyalkanoates (PHA) will become more relevant.

3.1 Microplastics – emergent contaminants in soil

MP have been detected in virtually any environmental compartment, including remote areas from civilization, such as in the deep sea, the Arctic, and glaciers in the Alps (*Bergmann* et al., 2019; *Scheurer* and *Bigalke*, 2018; *Allen* et al., 2019; *Woodall* et al., 2014). A predicted 80 % of ocean plastics originate from land (*Bläsing* and *Amelung*, 2018; *Rochman*, 2018). Thus, the land is likely a larger MP reservoir than the ocean. Mass flows of (micro-) plastics into soils are estimated to be 40 times larger than those into waters (*Kawecki* and *Nowack*, 2019).

Evidence for the presence of MP in the soil came late compared to the sea or freshwaters. In their study, Zubris and Richards (2005) tested whether synthetic fibers could act as indicators of sewage sludge (suggested by *Habib* et al., 1998). They examined soils where sewage sludge had been applied in the past and found that these soils contained synthetic fibers, thereby providing one of the first quantitative evidence of the presence of MP in soils and establishing the entry of MP to the soil via sewage sludge. Despite these early findings, awareness of MP in soil remained low. In 2012, *Rillig* (2012) called for increased research on plastic pollution in soils, and as of 2016, the first studies on MP interactions with earthworms appeared (*Huerta Lwanga* et al., 2016; *Rillig* et al., 2017b). Between 2012 and 2021, only 10 % of research articles on MP pollution dealt with terrestrial ecosystems (Joos and Tender, 2022). Thus, compared to aquatic ecosystems, research on the effects of MP occurrence, fate, and behavior in terrestrial ecosystems is still in its infancy (*Bläsing* and *Amelung*, 2018; *Horton* et al., 2017).

MP-containing sewage sludges, organic fertilizers (composts and digestates), and plastic mulches are significant input pathways of MP into agricultural soils (*van Schothorst* et al., 2021; *Corradini* et al., 2019; *van den Berg* et al., 2020; *Braun* et al., 2021; *Gui* et al., 2021; *Wang* et al., 2021; *Weithmann* et al., 2018). In addition, MP can also enter agricultural soils through wet and dry atmospheric deposition, littering, and runoff from nearby roads (*Bläsing* and *Amelung*, 2018; *Allen* et al., 2019; *Kernchen* et al., 2022).

Detecting MP in soils is challenging because MP must be isolated from the soil matrix; organic and mineral particles that adhere to MP must be removed (*Möller* et al., 2020; *Bläsing* and

Amelung, 2018). Several suitable methods exist depending on the research question, i.e., whether mass content or particle number of MP are of interest (*Möller* et al., 2020). For example, *Möller* et al. (2022) have developed a robust method for determining particle number, plastic type, size, and shape of MP in soil samples of up to 250 g. MP is analyzed by micro-Fourier transform infrared spectroscopy (FTIR) after efficient removal of mineral and organic fractions of soil samples, including density separation and oxidative-enzymatic digestion. For example, the method from Dümichen et al. (2017) is available to obtain mass-based MP concentrations of soil samples, which allows the analysis of a sample of 100 mg without prior purification using thermal extraction desorption gas chromatography-mass spectrometry (TED GC-MS).

A recent review stated that data on MP concentrations in soils are still scarce, mainly due to few studies conducted (n = 23 by 2020) and the use of detection methods with varying quality (*Büks* and *Kaupenjohann*, 2020). According to this review, the most comprehensive data existed for agricultural soils, where median concentrations of MP amounted to 1,200 particles kg^{-1} (25^{th} and 75^{th} percentiles: 88 and 2,830 particles kg^{-1}) and 1.7 mg kg^{-1} (0.3 and 2.8 mg kg^{-1}) (*Büks* and *Kaupenjohann*, 2020). However, these estimates are exclusively based on fields with the application of sewage sludges and plastic mulches as MP entry pathways. Only recently, *van Schothorst* et al. (2021) reported an MP load of 888 particles kg^{-1} for soil with 7 – 20 years of compost application.

3.2 Soil organisms and functions

Soils harbor various soil organisms, including plants, animals, and microorganisms (*Joos* and *Tender*, 2022; *Orgiazzi* et al., 2016). Soil organisms are organized in the soil food web, where they continuously interact with one another and their abiotic environment, forming the soil habitat (*Voroney* and *Heck*, 2015). Soil organisms mediate crucial soil processes that maintain numerous pivotal ecosystem services for plant growth and food production (*Orgiazzi* et al., 2016). The basis of the soil food web are microorganisms (mainly bacteria and fungi) by transforming and decomposing soil organic matter and plant litter, thereby driving C and nutrient cycling, of which the agents are soil microbial enzymes (*Burns* et al., 2013). Central actors in the soil food web are nematodes, which occupy different niches in the soil habitat and various positions in the soil food web on different trophic levels (*Orgiazzi* et al., 2016; *Coleman* and *Wall*, 2015; *Yeates* and *Bongers*, 1999). Nematodes have a wide range of diets, e.g., feeding on bacteria, fungal hyphae, and plant litter, thereby maintaining essential soil functions such as regulating matter cycles (*Yeates* et al., 1993; *Bardgett* et al., 1999).

Various anthropogenic stressors, such as climate change, chemical pollution, and land use intensification, threaten soil functions (*Orgiazzi* et al., 2016; *Schaeffer* et al., 2016; *Joos* and *Tender*, 2022). Growing evidence of the occurrence of MP in soil and the harmful effects of MP on soil organisms have raised concerns that MP could represent an additional stressor to soil functions (*Chae* and *An*, 2018; *Souza Machado* et al., 2018). If this proves to be accurate, not only weathering MP in aquatic ecosystems but also terrestrial ecosystems are a planetary boundary threat related to "chemical pollution and novel entities." Compared to other contaminant classes, e.g., pesticides and hydrocarbons, MP are distinct because of their potentially more complex ways and multiple ways MP can interact with the soil habitat and organisms (*Helmberger* et al., 2020). In addition to their physicochemical diversity, MP come as particles, i.e., MP have surfaces enabling interactions with other chemicals and organisms (*Helmberger* et al., 2020; *Rochman* et al., 2019).

3.3 Biodegradation of microplastics in soil

The colonization of MP is the first step of MP biodegradation (*Sander*, 2019). In the next step, hydrolytic enzymes produced by microorganisms initiate the depolymerization of MP. Then, the resulting mono- and oligomers are taken up and metabolized and either converted to CO_2, H_2O, and energy (under aerobic conditions) or microbial biomass (*Agarwal*, 2020). Biodegradation of MP depends on polymer-specific properties (e.g., hydrolyzable vs. non-hydrolyzable, chemical composition, and size), soil physicochemical conditions (e.g., water content, temperature, and pH), and soil microbial abundance, composition, and activity (*Kliem* et al., 2020; *Krueger* et al., 2015).

Most conventional polymers (polyethylene (PE), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC)) are recalcitrant to hydrolysis. The depolymerization of these polymers can only occur through UV- and heat-catalyzed chemical oxidation of the polymer's C backbones (*Krueger* et al., 2015; *Zhang* et al., 2021; *Agarwal*, 2020). Microorganisms can take up the resulting polymer fragments if smaller than 600 Da or between 10 and 50 C atoms (*Lehmann* and *Kleber*, 2015; *Restrepo-Flórez* et al., 2014). However, in soil, PE biodegrades exceptionally slowly. For instance, *Albertsson* (1980) determined a weight loss of PE between 0.1 – 0.4 % after 800 days in soil. Due to this low biodegradability, a standard on the biodegradability of plastics in soil proposes PE as a negative control (*DIN EN ISO 17556:2012 - 12*, 2012).

In contrast, biodegradable polymers have functional groups that offer depolymerization targets for biotic and abiotic hydrolysis (*Zhang* et al., 2021). Hydrolytic enzymes can catalyze the

depolymerization of biodegradable MP. For example, enzymatic hydrolysis through lipases and cutinases controls the biodegradation of PBAT in the soil (*Zumstein* et al., 2017; *Zumstein* et al., 2018). In addition to enzymatic hydrolysis, depolymerization of PLA can also occur via chemical hydrolysis (*Richert* and *Dąbrowska*, 2021; *Karamanlioglu* et al., 2014). Many studies have reported the potential of microorganisms isolated from soil or composts to biodegrade PLA, PBAT, and their blends (*Chomchoei* et al., 2011; *Karamanlioglu* et al., 2014; *Teeraphatpornchai* et al., 2003; *Jia* et al., 2021). These polymers have also proven biodegradable under laboratory settings in different environments, including in soils (*Zumstein* et al., 2018; *Emadian* et al., 2017; *Chomchoei* et al., 2011; *Ren* et al., 2019; *Saadi* et al., 2013; *Palsikowski* et al., 2018; *Freitas* et al., 2017). However, the biodegradation of films of these polymers in soil was slow and incomplete within the studied periods (*Palsikowski* et al., 2018; *Freitas* et al., 2017; *Saadi* et al., 2013; *Muniyasamy* et al., 2016). The highest C mineralization of only 21 % after 180 days was observed for PBAT (*Palsikowski* et al., 2018). One study reported that PLA films persisted for 11 months in Mediterranean soil under field conditions with minimal visible disintegration (*Rudnik* and *Briassoulis*, 2011). Yet, based on such visual evaluation, it remains unclear whether PLA was actually biodegraded or only fragmented and not instead contributed to the *in-situ* formation of MP (*Helmberger* et al., 2020; *Agarwal*, 2020). However, all findings cited here are based on investigations with films. MP could show better biodegradability because MP represent a more accessible form for microorganisms due to a higher surface-to-volume ratio than films (*Albertsson*, 1978; *Sander* et al., 2019). In addition, soil moisture is an important driver of microbial activity and biodegradation in soil (*Krueger* et al., 2015). For example, dry soils reduce enzymatic hydrolysis and biodegradation of polyhydroxyalkanoates (PHA) (*Meereboer* et al., 2020). Systematic investigation of the factors of size and soil moisture content will reveal potential constraints of biodegradation of PLA/PBAT-MP in soil.

3.4 Implications of microplastics for soil microorganisms

Emerging evidence shows that soil microorganisms colonize plastic surfaces (*Huang* et al., 2019; *Yi* et al., 2021; *Zhang* et al., 2019; *Rüthi* et al., 2020; *Bandopadhyay* et al., 2020; *MacLean* et al., 2021). In these studies, the composition of the plastics-associated microbiome showed lower diversity than bulk soil, while certain microbial groups enriched on the plastic surfaces. Compared to conventional PE, biodegradable plastics showed a more distinct microbial community (*Rüthi* et al., 2020; *Bandopadhyay* et al., 2020). One study found pioneer bacteria

such as autotrophs enriched on PE surfaces (*MacLean* et al., 2021). The authors proposed that the autotrophs might have contributed to surface erosion, thereby enabling biodegradation through possible subsequent microbial consortia. MP could act as evolutionary catalysts by selecting a plastics-associated microbiome (*Rillig* et al., 2019). In analogy to other biologically relevant soil spheres, e.g., the rhizosphere (*Beare* et al., 1995), MP could form a specific microbial habitat in soil. This microbial habitat would comprise the MP surface and the surrounding soil affected by MP – the plastisphere.

By forming a plastisphere and selecting plastics-associated microbiomes, MP can affect the soil microbial composition and abundance (*Rillig* et al., 2019). Additionally, MP-induced alterations in soil physicochemical properties, including bulk density, porosity, aggregation, water holding capacity, pH, and nutrient availability, could indirectly contribute to changes in soil microbial composition and abundance (*Zhang* et al., 2021). Shifts in soil microbial composition and abundance may imply that microbial processes involved in biogeochemical cycling could also change. A recent review concluded that the few studies on the effects of conventional MP on activities of enzymes involved in the biogeochemical cycling of C, N, and P, have not yet shown a consistent pattern (*Joos* and *Tender*, 2022; *Zhang* et al., 2021). For example, PE and PVC increased fluorescein diacetate hydrolase but decreased urease and phosphatase activities in the soil (*Fei* et al., 2020). In a field study, PE increased α- and ß-glucosidase activities in the soil (*Lin* et al., 2020). In the case of biodegradable MP, biodegradation processes such as the depolymerization and the utilization of MP-C are likely to affect microbial abundance and activity, C, and nutrient turnover (*Rillig* et al., 2021). Recent evidence supports this, as poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) formed hotspots of increased microbial abundance and activity and enhanced activity of ß-glucosidase and leucine aminopeptidase in the soil (*Zhou* et al., 2021). The authors suggested that these PHBV-induced stimulations in C and nutrient turnover could accelerate the decomposition of native soil organic matter. Other biodegradable polymers such as PLA and PBAT have also been shown to create a plastisphere in soil (*Rüthi* et al., 2020) and therefore also have the potential to alter C and nutrient cycling in soil.

3.5 Implications of microplastics for the soil fauna

Other interactions may occur on upper trophic levels of the soil food web. Earthworms (*Rillig* et al., 2017b; *Huerta Lwanga* et al., 2017) and springtails (*Maaß* et al., 2017) were found to translocate MP in soils, thereby contributing to the exposure of other soil-dwelling species to MP. The above-mentioned MP-induced shifts in soil microbial abundance and composition

could affect soil organisms, e.g., nematodes, which feed on soil microorganisms. In addition, MP are sufficiently small to be taken up by soil fauna as a function of the species-specific size-to-mouth ratio and thus could enter the soil food web (*Chae* and *An*, 2018; *Horton* et al., 2017). Once having entered the organism, MP can generally cause either physical impacts (particulate hazard) or chemical toxicity (indirect hazard), or a combination of both (*Horton* et al., 2017). First studies on the responses of nematodes towards MP proved that nematodes ingested conventional MP fragments and beads, and this was associated with negative impacts on their intestinal damage, a decrease in survival rates, lifespan and body length, oxidative stress as well as motor behavior (*Lei* et al., 2018a; *Lei* et al., 2018b). Likely, nematodes also ingest biodegradable MP, which could negatively affect their biological functions, but there is no evidence.

4 Aim and objectives

Concerns that MP pose a threat to the planetary boundary within the context of "chemical pollution and novel entities" (*Arp* et al., 2021) necessitate revealing potential ecological risks of MP in soils. This applies especially to agricultural soils, given their fundamental role in food production and safety (*Rillig* et al., 2017a). My thesis aimed to assess the risk of conventional and biodegradable MP compromising soil organisms and processes in agricultural soils.

The basis of the risk assessment was to establish whether organisms are exposed to MP in agricultural soils. Input rates of MP into the soil and transport and transformation processes of MP in the soil determine long-term exposure levels of soil organisms towards MP (*Sander* et al., 2019). Therefore, it is vital to learn background concentrations and the fate of MP, including biodegradation and persistence of MP, in the soil.

MP can form a specific habitat, biodegrade, and induce soil physicochemical alterations in the soil (*Rillig* et al., 2021). Therefore, MP threaten soil microorganisms and critical microbial processes, e.g., the decomposition of soil organic matter. Since soil microorganisms play crucial roles in maintaining C and nutrient cycling, it is necessary to elucidate the potential of MP to affect soil microbial abundance and processes.

Changes in soil microbial abundance and composition could affect soil organisms on other trophic levels feeding on soil microorganisms. But also, MP are sufficiently small to be ingested by soil organisms associated with chemical or physical hazard of MP, resulting in adverse effects on biological functions (*Horton* et al., 2017). Since nematodes occupy central positions in the soil food web (*Yeates* et al., 1993; *Bardgett* et al., 1999), it is urgently needed to clarify the potential of MP to enter the food web via nematode uptake and the effects of MP on biological functions of nematodes.

Controlled conditions in the laboratory allow us the study of specific factors of MP biodegradation and effects of MP (e.g., plastic type, particle size, soil moisture) on soil organisms, while other factors are excluded (e.g., constant temperature). Under field conditions, different factors (e.g., temperature, soil moisture, UV light) vary and interact over time, which may influence the behavior of MP in the soil. Therefore, field studies play a critical role in validating the results obtained from the laboratory concerning the relevance to the field.

My objectives were to study (1) MP background levels, (2) the potential biodegradation, and (3) the fate of MP in agricultural soil, as well as to investigate (4) possible effects of MP on soil microbial abundance, composition, and C turnover under both controlled and field conditions,

and (5) the potential of MP to be ingested by nematodes and to affect biological functions of nematodes.

5 General approach

To achieve my objectives, I combined a microcosm study ("Hydrolyzable MP in soil – low biodegradability but formation of a specific habitat?"), with a field study ("Microplastics persist in an arable field but do not affect soil microbial biomass, enzyme activities, and crop yield."), and a nematode study ("Microplastics effects on reproduction and body length of the soil-dwelling nematode *Caenorhabditis elegans*").

In the microcosm study, the influence of the factors of plastic type (conventional and biodegradable), particle size, and soil moisture on biodegradation of MP in soil and on effects on soil microorganisms was examined. MP biodegradation was evaluated by quantifying the mineralization of C-derived MP (MP-C), calculated as the difference of CO_2 released from MP-amended soil compared to CO_2 released from MP-free soil over 230 days at 25 °C (*DIN EN ISO 17556:2012 - 12*, 2012). Possible effects of MP on soil organisms and their processes were assessed based on soil microbiological indicators. These included phospholipid fatty acids (PLFAs) as biomarkers for the abundance and composition of the main soil microbial groups and activities of C cycling enzymes that drive the decomposition of differently complex substances as proxies for C turnover. In an innovative approach, enzyme activities of individual MP particles, which were extracted from the soil after 230 days, were measured. This aimed to better understand the role of MP as an interface for microbial processes in the soil, such as the activity of lipases that can trigger the depolymerization of biodegradable MP with ester bindings.

The field study was conducted on conventionally managed agricultural soil at the research station Heidfeldhof (University of Hohenheim). In the past, the field was not treated with plastic mulches, organic fertilizers, or sewage sludges. Thus MP via these entry pathways could be ruled out. Before the start of the experiment, background concentrations of MP were analyzed. A randomized complete block design was leveraged to study the main effects of MP and organic fertilizers (digestate and compost) and their interaction effects on soil microbiological indicators (microbial biomass C and soil enzymes) over 1.5 years. The fate of MP in the field was assessed by comparing MP concentrations in soil at the beginning of the experiment with those after one month and 17 months. Particle-based MP concentrations in the soil were determined using the recently developed method by *Möller* et al. (2022).

In the nematode study, ecotoxicological tests with nematodes exposed to MP feed suspensions on agar plates were carried out. This approach enabled the study of the ingestion of Nile-red stained MP particles through the ecotoxicological model organism *Caenorhabditis elegans*

using fluorescence microscopy. In a combined reproduction and body length assay, the importance of the toxicity factors of plastic type and concentration on *C. elegans* were studied.

MP are diverse mixtures of multiple polymers and chemical compounds with different structural properties (*Bank* and *Hansson*, 2019; *Hartmann* et al., 2019). My thesis could not cover this diversity. Based on their potential biodegradability, two MP types were selected for my studies: the conventional (non-hydrolyzable) low-density-polyethylene (LDPE) and the biodegradable (hydrolyzable) mix polymer consisting of poly(lactic acid) and poly(butylene adipate-co-terephtalate) (PLA/PBAT).

LDPE is obtained by polymerizing ethylene under high pressure (*Kumar Sen* and *Raut*, 2015). Roughly 2 % of the carbon atoms are branched, which gives it a low density. Due to its chemical inertness and flexibility even at low temperatures, it is excellent as a packaging material and plastic bags (*Kumar Sen* and *Raut*, 2015). High hydrophobicity, stable C backbones, molecular weight (> 30 kDa), and non-hydrolyzability are constraints for the biodegradation of LDPE (*Restrepo-Flórez* et al., 2014; *Krueger* et al., 2015; *Kumar Sen* and *Raut*, 2015).

PBAT is a fossil-based synthetic polymer obtained from polycondensation reactions of 1,4-butanediol, adipic/terephthalic acids, and butylene adipate (*Ferreira* et al., 2019). The properties of PBAT are similar to that of LDPE, making it suitable for film applications such as organic waste bags, mulch films, and shopping bags (*Künkel* et al., 2010). PLA is an entirely biobased thermoplastic polymer (*Folino* et al., 2020). PLA is produced through fermentation of unrefined dextrose, i.e., glucose, derived from corn or starch to lactic acid, followed by chemical conversion to lactide (cyclic dimer of lactic acid) and ring-opening polymerization (*Künkel* et al., 2010). Similar properties like polystyrene, such as stiffness, make it suitable for producing plastic bottles, cups, and stiff packages. From a sustainable viewpoint, it is desirable to enhance the proportion of biobased materials such as PLA (*Muniyasamy* et al., 2016). For this purpose, PBAT and PLA can be combined via blending, which reduces the stiffness of PLA and increases its flexibility, making it feasible for film applications (*Künkel* et al., 2010). Blends of PLA and PBAT have become the most common biodegradable plastic materials for the production of compostable waste bags and plastic mulch films (*Künkel* et al., 2010; *Bandopadhyay* et al., 2020). Based on the applications of LDPE and PLA/PBAT, the presence of these polymers as plastic residues (MP) from organic fertilizers and plastic mulches in agricultural soils is a realistic scenario.

MP was provided by the “Institut für Kunststofftechnik”, University of Stuttgart, and produced by grinding polymer through a cryomill (refer to the Material & Methods sections of the studies

for details). Grinding resulted in irregularly shaped MP. None of the polymers contained additives because the aim was to study the sole effects of the polymers. The PLA/PBAT blend composition was 85/15 % w/w in the microcosm study and 80/20 % w/w in the field and nematode studies. During the conception phase of the experiments, not yet robust estimates for MP loads in agricultural soils did exist. Thus, the choice of concentration ranges used in the studies was based on standards such as the DIN ISO EN 17556 (*DIN EN ISO 17556:2012 - 12,* 2012) and literature (*Lin* et al., 2020; *Lei* et al., 2018b).

6 Hydrolyzable microplastics in soil – low biodegradation but formation of a specific habitat?

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6.1 Abstract

Microplastics (MP, plastic particles between 0.1 and 5000 µm) contaminate agricultural soils through the application of organic fertilizers, sewage sludge, and plastic mulch. MP surfaces and the MP-soil interface provide specific habitats for soil microorganisms – the plastisphere. Microorganisms in the plastisphere may benefit from utilizing MP as a carbon (C) source. Hydrolyzable MP with ester bonds are susceptible to enzymatic depolymerization by hydrolysis. In a microcosm experiment, we investigated MP biodegradation of small and large (< 0.5 mm and 0.5 – 2 mm respectively), hydrolyzable (a poly(lactic acid)/poly(butylene adipate-co-terephthalate) blend, PLA/PBAT) and non-hydrolyzable (low-density polyethylene, LDPE) polymers, and the effects of MP on microorganisms in dry and wet MP-amended soil. MP affected neither abundance and composition of the main soil microbial groups (fungi, Gram-negative, and Gram-positive bacteria), specific activities of ß-glucosidase, ß-xylosidase, lipase, and phenoloxidase, nor respiration in MP-amended soil. Only large PLA/PBAT particles in dry soil were significantly mineralized (15.4 % of initial PLA/PBAT-C after 230 days). PLA/PBAT mineralization coincided with enhanced lipase and ß-glucosidase activities on the surfaces of individual PLA/PBAT particles extracted from the soil after incubation (compared to LDPE and non-incubated PLA/PBAT particles). We detected cracks on the surfaces of PLA/PBAT particles using scanning electron microscopy, indicating initiation of MP biodegradation, presumably due to depolymerization by lipases. Results suggest that the PLA/PBAT plastisphere is a polymer-specific habitat for lipase-producing soil microorganisms. Our study demonstrates that analyzing biogeochemical interactions within polymer-specific plastispheres is essential to assess MP fate and their impacts on microbially-driven soil processes.

6.2 Introduction

Contamination of soils with microplastics (MP) is an understudied environmental threat (Helmberger et al. 2020; Rillig 2012). MP are particles between 100 nm and 5 mm in size, variable in shape (Souza Machado et al. 2018), and can originate from numerous synthetic polymers (> 5300, Jacquin et al. 2019). Agricultural soils receive substantial loads of MP through the application of MP-contaminated compost and sewage sludge, plastic mulch, and atmospheric deposition of MP (Zhang et al. 2021) and contain on average 1200 MP particles kg^{-1} and ~ 2 mg MP kg^{-1} (Büks and Kaupenjohann 2020).

Soil microorganisms play important roles in soil processes such as the transformation and decomposition of organic matter (Orgiazzi et al. 2016). Carbon (C) and nutrient cycling in soil

are mediated by extracellular enzymes produced by microorganisms (Burns et al. 2013). For example, ß-glucosidases and ß-xylosidases catalyze the hydrolysis of cellulose oligomers to glucose and xylan chains to xylose, and phenoloxidases initiate the decomposition of phenolic compounds (German et al. 2011; Kandeler 2015). In addition, soil microorganisms can mediate the biodegradation of MP in the soil through specific enzymes, then use the MP-derived C for growth and energy (Krueger et al. 2015; Ng et al. 2018; Rillig et al. 2021). MP biodegradation in soil generally proceeds via (1) microbial colonization of the MP surface, (2) depolymerization of MP to mono- and oligomers (< 50 C atoms with molecular weight < 600 Da (Lehmann and Kleber 2015; Restrepo-Flórez et al. 2014)) via enzymatic hydrolysis, and (3) subsequent microbial uptake and metabolism of MP-derived mono- and oligomers (Agarwal 2020; Sander 2019).

Microbial communities on plastic surfaces typically differ from the adjacent soil microbiome and exhibit reduced diversity compared to soil (Bandopadhyay et al. 2018, 2020; Huang et al. 2019; Rüthi et al. 2020; Yi et al. 2021; Mengjun Zhang et al. 2019; Zhou et al. 2021). Thus, analogous to other biologically relevant spheres in soil, such as the rhizosphere (Beare et al. 1995), the plastisphere in soil forms a new type of microbial habitat encompassing MP surfaces and the adjacent soil influenced by MP (Rüthi et al. 2020; Zhou et al. 2021). Changes in microbial composition and abundance may be due to the use of MP as a substrate by specific microorganisms (Rillig et al. 2019). Additionally, MP may indirectly affect microbial composition and abundance through changes in soil physicochemical properties, such as bulk density, porosity, aggregation, electrical conductivity, water holding capacity, pH, and nutrient availability (Zhang et al. 2021). In turn, changes in the soil microbial community may influence microbially-driven processes such as C and nutrient cycling (Rillig et al. 2021). However, responses of C cycling enzymes in soil and microbial activity to MP have not yet shown a clear pattern in soil (Xu et al. 2020). The few existing studies are inconclusive and report inhibitory, but also stimulatory MP effects on enzyme activities (Fei et al. 2020; Huang et al. 2019; Lin et al. 2020; Wang et al. 2020). Consequently, further research is needed to investigate such specific microbial processes in the plastisphere.

Specifically, investigating the secretion of hydrolytic enzymes in the plastisphere that can initiate MP biodegradation is crucial to understanding the fate of MP in soil, given that enzymatic hydrolysis has been recently reported as the rate-controlling step in plastics biodegradation (Sander 2019; Zumstein et al. 2017, 2018). Typical MP types found in agricultural soils, e.g., polyethylene, polystyrene, and polypropylene (e.g., Piehl et al. 2018), are not susceptible to enzymatic hydrolysis (non-hydrolyzable). Depolymerization of these MP

can only occur after heat- and UV-stimulated chemical oxidation of the polymers' backbones to enable steric accessibility by extracellular hydrolytic enzymes (Krueger et al. 2015). In contrast, hydrolyzable plastics contain specific functional groups that can be cleaved by specific enzymes. For example, lipases, esterases, and cutinases can catalyze the depolymerization of polyesters by cleaving their ester bonds (Marten et al. 2005; Meereboer et al. 2020; Teeraphatpornchai et al. 2003; Tokiwa and Calabia 2007; Zumstein et al. 2017). Examples of polyesters are the aliphatic poly(lactic acid) (PLA) and poly-hydroxy-butyrate (PHB), and the aromatic poly(butylene adipate-co-terephthalate) (PBAT) (Agarwal 2020). PLA and PBAT and their blends are used to replace conventional low-density polyethylene (LDPE) mulch films and trash bags (Bandopadhyay et al. 2018; Musiol et al. 2018; Künkel et al. 2016; Sander et al. 2019) and thus may end up in agricultural soils via the application of plastic mulches and organic fertilizers. PHB occurs naturally in soils in the form of lipids in soil bacteria (Mason-Jones et al. 2019). While PHB biodegrades at a rate of up to 98 % in soil, the biodegradation of PLA and PBAT as well as their blends in the soil is typically slow and their degradation pathways, such as through enzymatic hydrolysis, are not well understood (Bettas Ardisson et al. 2014; Emadian et al. 2017; Freitas et al. 2017; Palsikowski et al. 2018; Weng et al. 2013).

The typical slow degradation of PLA and PBAT may be linked to environmental constraints (Agarwal 2020). Soil moisture is a crucial driver of microbial activity and biogeochemical processes as well as for the biodegradation of MP in soil (Kliem et al. 2020; Krueger et al. 2015). For instance, hydrolysis and biodegradation of polyhydroxyalkanoates (such as PHB) typically slow down in dry soils (Meereboer et al. 2020). Additionally, the particle size of MP determines the specific surface area accessible for soil microorganisms and enzymes and can control MP biodegradation (Sander et al. 2019; Yuan et al. 2020). However, experimental evidence of the influence of soil moisture and particle size on MP biodegradation is rare and further systematic research is needed.

To better understand the fate of MP and assess the impacts of MP on C cycling in soil, we studied microbial interactions of MP-soil mixtures (MP-amended soil) and of individual MP particles with soil microorganisms in a microcosm experiment. We investigated the biodegradability of hydrolyzable and non-hydrolyzable MP (PLA/PBAT and LDPE) in two different size fractions and the potential effects of MP on microorganisms in dry and wet soil. We hypothesized that (1) only PLA/PBAT will be mineralized, while LDPE persists, (2) due to biodegradation of PLA/PBAT, soil microorganisms respond more strongly to PLA/PBAT than to LDPE, and (3) surfaces of individual PLA/PBAT particles exhibit morphological changes and enhanced activities of specific hydrolytic enzymes (lipases). We expected that

interactions of soil microorganisms with MP-amended soil and MP particles are strongest in wet soil (i.e., non-limiting microbial activity) and small MP particle size (i.e., high specific surface area).

6.3 Materials and methods

Microplastics preparation and characteristics

As hydrolyzable MP we used a blend of the polymers poly(lactic acid) (PLA; Ingeo™ Biopolymer 7001D, NatureWorks LLC, Minnetonka, MN, United States) and poly(butylene adipate-co-terephthalate) (PBAT; Ecoflex® F Blend C1200, BASF SE, Ludwigshafen, Germany) with a mixing ratio of 85/15 % w/w. The PLA/PBAT blend was compounded by extrusion of PLA and PBAT pellets using a twin-screw extruder without using any additives at the Institut für Kunststofftechnik (University of Stuttgart, Stuttgart, Germany). Low-density polyethylene (LDPE; Lupolen 2420 H, LyondellBasell Industries N.V., Rotterdam, Netherlands) served as conventional, non-hydrolyzable MP. Polyhydroxybutyrate (PHB) was used as a positive control in the biodegradation test as suggested in DIN EN ISO 17556:2012 - 12 (2012) and was purchased from Biomer (Krailling, Germany) as a fine white powder. Defined MP particle size fractions were obtained by grinding frozen polymer pellets (liquid nitrogen, –196 °C) with a speed rotor mill (Pulverisette, Fritsch GmbH, Idar-Oberstein, Germany). The ground particles were fractionated with stainless steel sieves to obtain two particle size fractions of < 0.5 mm (small) and 0.5 – 2 mm (large). The C content of the MP (n = 3) was measured with an element-analyzer (EA, Euro EA 3000, Euro Vector, Milan, Italy) and was 85.5 ± 0.3 % (mean ± SD) for LDPE, 58.3 ± 2.2 % for PLA/PBAT, and 55.6 ± 0.1 % for PHB.

Static image analysis was used to determine MP particle size and shape. For details we refer to Supplementary Information 1 Particle size and shape distributions. No meaningful differences in particle size were observed between plastic types in the 0.5 – 2 mm fraction, while there was a small difference in the fraction of < 0.5 mm between plastic types (Supplementary figures Table S6-1; Fig. S6-1). The median particle size was 0.051 mm (IQR: 0.092 mm) for LDPE < 0.5 mm, 0.024 mm (IQR: 0.106 mm) for PLA/PBAT < 0.5 mm, 0.806 mm (IQR: 0.459 mm) for LDPE 0.5 – 2 mm, and 0.813 mm (IQR: 0.325 mm) for PLA/PBAT 0.5 – 2 mm. PHB reference particles were considerably smaller, with a median size of 0.008 mm (IQR: 0.010 mm).

Based on visual inspection and a combination of three different shape descriptors, i.e., sphericity, elongation, and solidity, most particles of both LDPE and PLA/PBAT could be

characterized as irregularly shaped, while some of the particles had a fibrous shape (Table S6-1; Fig. S6-2; Fig. S6-3; Cole 2016; Hartmann et al. 2019). All PHB particles were irregularly shaped (Table S6-1).

Soil sampling and characteristics

The soil was randomly sampled (0 - 20 cm, *Ap* horizon) from an agricultural field of the research station Heidfeldhof (University of Hohenheim, central point of the field: 9°11´22.984´´ longitude, 48°43´11.137´´ latitude, EPSG: 4326, WGS 1984) in July 2018. We passed the soil through a 2-mm mesh stainless steel sieve and removed plant residues and organic material. We then adjusted the soil moisture content of the soil according to our experimental design and pre-incubated the soil at 25 °C for 2 weeks in a bucket with small holes to avoid significant soil moisture changes. Before use, soil moisture changes were compensated. The soil is classified as a Luvisol according to the world reference base for soil resources (FAO 2006) with silty loam texture (22.2 % clay, 75.1 % silt, 2.7 % sand). Soil pH (measured in 0.01 M $CaCl_2$) was 5.4. Total soil C and N were 1.18 % and 0.13 %, respectively. Soil microbiological data were calculated on a dry weight basis. Soil dry weight was determined by drying aliquots of soil samples for 24 h at 105°C.

The specific surface area of the soil was determined by the methylene blue (MB) titration method (Santamarina et al. 2002; Yukselen and Kaya 2006). In short, we suspended 2 g of dry soil in 200 mL deionized water and added 0.02 – 0.06 g of the cationic dye MB (121170.1608, AppliChem GmbH, Darmstadt, Germany). The MB-soil-suspensions (n = 3 per concentration level) were shaken for 2 h at 200 rpm and incubated overnight, allowing soil particles to settle. The next day, aliquots of 5 mL were centrifuged for 10 min at 1320 × g. The remaining MB concentration in the suspension was determined using a photometer (Synergy HTX multi-mode reader, Bio-Tek Instruments Inc., Winooski, VT, USA) by measuring the absorbance at 655 nm. To obtain the amount of MB absorbed into the soil, the remaining MB concentration was subtracted from the amount of MB added to the soil. The point of complete cation replacement (saturation) was identified visually (Yukselen and Kaya 2006) at a mass ratio of MB added to the soil of 0.02 (Fig. S6-4). The specific surface area of the soil was determined as 49.0 $m^2\ g^{-1}$.

Experimental design and incubation conditions

We mixed small (< 0.5 mm) and large (0.5 – 2 mm) LDPE and PLA/PBAT particles separately and homogeneously with dry (pF = 4) and wet soil (pF = 2), respectively, to obtain a final MP concentration of 1 mg MP g^{-1} dry soil (Table S6-2). MP-free soil was included as a control

treatment. Soil aliquots of 150 g were incubated in microcosms (0.75-L glass jars with airtight lids) at 25 °C in the dark and sampled after 104 and 230 days (four replicates per treatment, 48 microcosms in total). During incubation, anoxic conditions within the microcosms were prevented by allowing regular aeration for about 3 h; approximately every 3 days within the first 2 months and every 10 days thereafter. Oxygen limitation can be ruled out because PHB was completely mineralized (Table S6-3). In addition, water loss of soil was monitored gravimetrically and compensated monthly by adding approximately 1 mL sterile deionized water to the soil. Some microcosms were excluded from the data set due to erroneous handling during CO_2 measurements resulting in at least 2 – 3 replicates included in the final data evaluation (Table S6-2).

Abundance and composition of the main soil microbial groups

The abundance and composition of the main microbial groups (fungi, Gram-negative, and Gram-positive bacteria) were evaluated using phospholipid-derived fatty acids (PLFAs) as biomarkers (e.g., Hallama et al. 2021). PLFAs concentrations in soil were measured based on Frostegård et al. (1991). In short, we extracted PLFAs from 4 g fresh (~3.3 g dry soil at pF 2, ~3.6 g dry soil at pF 4) per sample with single-phase Bligh and Dyer reagent (mixing ratio of chloroform, methanol, and citrate buffer pH 4 of 1:2:0.8, v/v/v). In the first extraction step, 18.4 mL Bligh and Dyer reagent were used per 4 g fresh soil. Lipids were fractionated via solid-phase extraction using silica acid columns (0.5 g silicic acid, 3 mL, Varian Medical Systems, Palo Alto, California). PLFAs were then transformed into fatty acid methyl esters via alkaline methanolysis and quantified by a gas chromatograph (AutoSystem XL, Perkin-Elmer Inc., Norwalk, CT). We refer to Kramer et al. (2013) for a detailed method description.

PLFAs were assigned to microbial groups according to Kandeler (2015). As Gram-positive bacterial markers we used the PLFAs a15:0, i15:0, i16:0, i17:0, and as Gram-negative, cy17:0 and cy19:0. As a general bacterial biomarker, we used the PLFA 16:1ω7 and the PLFA 18:2ω6,9 as a biomarker for fungi. We added biomarkers of Gram-negative, Gram-positive, and general bacteria to obtain the sum of bacterial PLFAs. As a proxy for microbial biomass, we used the sum of microbial PLFAs. This included all biomarkers of bacterial and fungal markers presented here in addition to the unspecific microbial markers 14:0, 15:0, 16:0, 17:0, 18:0, and 20:0 as recommended by Joergensen (2022). Moreover, we included the PLFA 16:1ω5 in the calculation of the sum of microbial PLFAs which occurs in both bacteria and fungi (Olsson and Lekberg 2022), but we excluded the marker 20:4ω6,9,12,15 because it occurs

in the microfauna (Ruess and Chamberlain 2010). In addition to the microbial group markers, we evaluated the ratios of Gram-positive to Gram-negative bacterial and of fungal to bacterial markers. We are aware that we did not consider the specific markers (10Me16:0, 10Me17:0, 10Me18:0) of Actinobacteria (Joergensen 2022) and thus may have underestimated the Gram-positive bacteria as well as the sum of bacterial and microbial PLFAs. However, with our current method, we were not able to measure these.

Biodegradation of MP

Biodegradability of MP was estimated by measuring MP mineralization following DIN EN ISO 17556:2012 - 12 (2012) as the difference between CO_2-derived C released from MP-amended soil and from MP-free soil in relation to C initially input as MP to the soil (MP-C). CO_2 production from MP-free and MP-amended soil was determined by titration as described in Poll et al. (2010). Specific microbial respiration rates were obtained by normalizing CO_2 production rates at 104 and 230 days to the sum of microbial PLFAs in soil.

Potential enzyme activities

Enzyme activities in soil

Potential activities of the enzymes ß-glucosidase, ß-xylosidase, lipase, and phenoloxidase were measured using fluorometric and photometric methods (German et al. 2011; Marx et al. 2001; Floch et al. 2007). ß-Glucosidase and ß-xylosidase activities were measured as described in Kramer et al. (2013). Lipase activity was determined using a modified protocol according to Cooper and Morgan (1981). Substrates and standards were purchased from Sigma-Aldrich (St. Louis, MO, United States). Standard stock solutions of 5 mM 4-methylumbelliferyl (MUF, M1381) were obtained by dissolving MUF in methanol and deionized water (1:1). Standard working solutions (10 µM MUF) were prepared in 0.1 M Tris-HCl buffer pH 6.8 (lipases) or MES buffer pH 6.1 (ß-glucosidase and ß-xylosidase). For each soil sample, we prepared a standard curve with concentrations of 0, 0.5, 1, 2.5, 4, and 6 µM MUF in soil suspension aliquots and buffer. We included calibration curves in deionized water and buffer to determine the quenching factor according to German et al. (2011). Lipase substrate stock solutions (10 mM) were obtained by dissolving the substrates 4-methylumbelliferyl heptanoate (M2514) and oleate (75164) in dimethyl sulfoxide (D8418). Working solutions (1 mM) were prepared by adding sterile 0.1 M Tris-HCl buffer pH 6.8. Substrate solutions of ß-glucosidase and ß-xylosidase were prepared and the analytical procedure was carried out following Kramer et al. (2013).

Soil phenoloxidase activity was analyzed based on Floch et al. (2007) using 2,2′-azinobis-(-3 ethylbenzothiazoline-6-sulfononic acid) diammonium salt (ABTS, A1888, Sigma-Aldrich, St. Louis, MO, United States) as the substrate. ABTS can be oxidized by phenoloxidases to the blue-green-colored $ABTS^{\cdot+}$ radical cation, which can be measured photometrically (Floch et al. 2007; Koleva et al. 2001). ABTS stock and working solutions (10 and 2 mM) were prepared in modified universal buffer (MUB) pH 4. MUB was prepared by dissolving tris(hydroxymethyl)aminomethane (4855.2, Carl Roth GmbH + Co. KG, Karslruhe, Germany), maleic acid, citric acid, and boric acid in deionized H_2O and adjusting to pH 4 with 1M NaOH (Floch et al. 2007).

ABTS converts to $ABTS^{\cdot+}$ by reacting with potassium persulfate ($K_2S_2O_8$, 1.05091, Merck KGaA, Darmstadt, Germany) in a ratio of 2:1 (Koleva et al. 2001). As the standard, a $ABTS^{\cdot+}$ solution was prepared. First, 8 mM phosphate-buffered saline (PBS) pH 7.4 was prepared according to Koleva et al. (2001). Then, a 50 mM $K_2S_2O_8$ solution was prepared in PBS. To obtain the final 1 mM $ABTS^{\cdot+}$ standard solution, 1 mL from the 10 mM ABTS stock solution was thourougly mixed with 8.89 mL of the PBS solution and 0.11 mL of the $K_2S_2O_8$ solution. Mixing in this manner yields a small excess of $K_2S_2O_8$ in the standard solution, which promotes the conversion from ABTS to $ABTS^{\cdot+}$. Next, the $ABTS^{\cdot+}$ standard solution was incubated in the dark at room temperature overnight. The conversion of ABTS to $ABTS^{\cdot+}$ was verified by the presence of a clear extinction maximum at 414 nm using a photometer (Synergy HTX multi-mode reader, Bio-Tek Instruments Inc., Winooski, VT, USA). On each microplate, calibration curves were prepared with final concentrations of 0, 20, 40, 60, 80, and 100 µM $ABTS^{\cdot+}$ that were prepared in MUB pH 4.

To measure soil phenoloxidase activity, 0.4 g fresh soil was suspended in 50 mL deionized H_2O and dispersed through ultrasonication for 2 min at 50 J s^{-1}. Per sample, in triplicate, 100 µl soil suspension and 100 µL MUB pH 4 were pipetted onto one microwell. Then, 50 µl ABTS working solution was added. Also, controls without substrate were employed in triplicate (100 µl soil suspension and 150 µL MUB pH 4). The microplates were preincubated at 30 °C for 5 min, before absorbance was read by the photometer at a wavelength of 414 nm over 30 min at 3 min - intervals.

Soil enzyme activities were calculated based on German et al. (2011). Enzyme activities were then divided by the sum of all microbial PLFAs to obtain specific enzyme activities (nmol $nmol^{-1}$ PLFAs h^{-1}) (Kandeler and Eder 1993; Landi et al. 2000). For the comparison of enzyme activities in the soil to those on the surfaces of MP particles, we normalized soil enzyme activities to the specific surface area of the soil (nmol mm^{-2} dry soil h^{-1}).

Enzyme activities on MP particles

At the end of the experiment, we sampled LDPE or PLA/PBAT particles from the treatments with large MP particles. To separate the MP from the soil, we sieved the MP-amended soil by wet-sieving using stainless steel sieves (mesh size: 0.5 mm). For the enzyme assay, we analyzed 12 individual particles from each microcosm (36 – 48 individual particles per treatment) (Table S6-2; Fig. S6-5). Non-incubated particles served as controls.

We analyzed lipase activity because lipases, among other enzymes, may initiate PLA/PBAT biodegradation by hydrolysis of PLA/PBAT ester bonds, indicating the activity of potential PLA/PBAT degraders. In addition, we analyzed ß-glucosidase activity as an indicator of cellulose-degrading microorganisms. Enzyme activities of MP particles were measured using the methods described above with slight modifications. We used the same substrates as in the enzyme assay with soil, but in this case only the lipase substrate heptanoate. Substrate controls with no particles were included in the assay. To each microplate well containing one MP particle, we added 50 µl deionized water, 50 µl buffer (0.1 M Tris-HCl pH 6.8 for lipase and MES pH 6.1 for ß-glucosidase) and 100 µl substrate. Calibration curves were prepared in buffer and deionized water. Before pre-incubation at 30 °C for 30 min, the microplates were put in an ultrasonic bath for 5 min to ensure good contact of the MP particles to the substrate. Fluorescence signals were recorded with a measurement interval of 30 min over 3 h. Limit of detection (LOD) and quantification (LOQ) were estimated using functions from the R package envalysis 0.4 (Steinmetz 2020). LOD and LOQ were 0.018 and 0.103 µM MUF for lipase and 0.026 and 0.058 µM MUF for ß-glucosidase. If the MUF concentration changes of the samples during the measurement period were below the LOQ and LOD, we classified the entries as below LOQ and LOD and excluded these from data evaluation (Fig. S6-5).

Following the enzyme analyses, the MP particles were picked out of the microplates and their surface area was estimated with a digital 3D-microscope (VHX-7000 & VHX-S650E, KEYENCE CORPORATION, Osaka, Japan) using the depth composition function. To get a better estimate of the 3D surface area of the particles, we added the cross-sectional area of the particles to the estimated surface area, since the area of the side of the particles on which they were positioned was not accessible to the microscope's camera.

Microbial colonization and surface morphology of MP

Scanning electron microscopy was used to assess both microbial colonization of MP and morphological changes on the surfaces of MP. To investigate microbial colonization of MP, MP particles were first rinsed in water to remove loosely attached material, fixed with 2.5%

glutaraldehyde working solution (prepared from an aqueous 25 % glutaraldehyde stock solution, EM grade, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) in 1 × phosphate-buffered saline, then stored at –20 °C in EtOH/phosphate-buffered saline (50/50 % v/v) until dehydration in 2,2-dimethoxypropane for 10 min, followed by critical point drying with CO_2 (CPD020, Balzers Union, Balzers, Liechtenstein). Any biofilms formed on the particles can be assumed to sustain our preparation procedure (Kirstein et al. 2019; Mengjun Zhang et al. 2019). To study morphological MP changes, MP particles were picked from the soil with no further pre-treatment. Then, all samples were coated with gold-palladium. Images were taken with a scanning electron microscope using a secondary electron detector (ZEISS EVO 15, Carl Zeiss AG, Jena, Germany). For the study of pristine particles and morphological degradation, beam energy was set to 15 kV and beam current of 10 pA was used, while settings were 10 kV and 100 pA, when investigating biofilm formation. Panels were created using FigureJ 1.38 (Mutterer and Zinck 2013).

Data analyses

For statistical data analyses and visualization, we used the statistical software R 4.0.2 (R Core Team 2020) and RStudio 1.2.5042 (RStudio, Inc. 2020) with the following packages: broom 0.7.0 (Robinson et al. 2020), broom.mixed 0.2.6 (Bolker and Robinson 2020), lubridate 1.7.9 (Grolemund and Wickham 2011), svglite 1.2.3.2 (Wickham et al. 2020), patchwork 1.0.1 (Pedersen 2020), ggtext 0.1.0 (Wilke 2020), ggbeeswarm 0.6.0 (Clarke and Sherrill-Mix 2017), tidyverse 1.3.0 (Wickham et al. 2019), ggpubr 0.4.0 (Kassambara 2020), flextable 0.5.10 (Gohel 2020), and MPA 1.1.0 (Schnepf 2021). Minor optical modifications (coloration) to the figures were made with Inkskape 1.0.1 (Inkscape Project 2020). Reproducible R codes and data sets generated for this study are available from Mendeley Data (https://doi.org/10.17632/22jwmgvjcr.3).

We tested significant differences between cumulative CO_2-derived C released from MP-amended soil and MP-free soil by performing two-tailed Dunnett's tests for each soil moisture level to determine mineralization of MP-C (Fig. S6-6b, Table S6-3).

To evaluate the specific enzyme activities, specific microbial respiration rates, and PLFAs markers, we fitted linear mixed effect models to the data matching our experimental design (Table S6-2). The treatment structure consisted of the factor soil moisture (*pF*) crossed with a dummy factor *ConVSTrt* (control versus treatment) (Piepho et al. 2006). Nested within *ConVSTrt*, we crossed the factors *PlasticType* and *ParticleSize*. We crossed *Timepoint* as a repeated measures factor with all other treatment terms and allowed a random intercept for the

randomization unit (microcosm ID) (Piepho et al. 2004). The model was fitted to the data using the "lmer" command from the lme4 1.1-23 package (Bates et al. 2015). As suggested by Forstmeier and Schielzeth (2011), we did not simplify the models, but used the full models only. Next, we conducted ANOVAs, approximating degrees of freedom with the Kenward-Roger method (Kenward and Roger 1997) provided in the lmerTest 3.1-2 package (Kuznetsova et al. 2017). Based on visual inspection with diagnostics plots (Harrison et al. 2018), we confirmed the model assumptions.

Lipase activity data on MP surfaces was evaluated using a linear mixed effect model with the "lme" command from the nlme 3.1-148 package (Pinheiro et al. 2021). We crossed the factors plastic type (*PlasticType)* and soil moisture (*pF*) and added microcosm (*ID*) as a random factor. Log-transformed lipase data met the model assumptions of normality and variance homogeneity. To improve the model, we fitted a variance structure per stratum to the model. Next, we conducted an ANOVA. We could not statistically analyze ß-glucosidase activity on MP particles because most of the data entries were below the limit of quantification (Fig. S6-5). Using $p < 0.05$ as the cut-off level, we identified statistically significant terms in the ANOVAs and compared estimated marginal means according to our hypotheses using functions of the emmeans 1.5.0 package (Lenth 2020).

6.4 Results

MP biodegradability in soil

We evaluated the biodegradability of MP in soil by the difference in CO_2 released from MP-amended compared to MP-free soil expressed as the relative amount of mineralized MP-C initially added to the soil. Within 230 days, only large PLA/PBAT particles were significantly mineralized in dry soil (15.4 %, $t_{12} = 3.90$, $p = 0.009$). C mineralization of small PLA/PBAT particles in dry soil was 10.7 %, but this was not significantly different from 0 (Fig. 6-1, Table S6-3). PLA/PBAT particles were not mineralized in wet soil (Fig. 6-1, Table S6-3). In contrast, C of the reference polymer PHB was almost completely mineralized in dry soil (84.3%, $t_{12} = 19.28$, $p < 0.001$) and completely mineralized in wet soil (130.7 %, $t_{12} = 43.76$, $p < 0.001$). We did not observe significant mineralization of small PLA/PBAT particles or of LDPE particles (Fig. 6-1, Table S6-3). Wet soil amended with LDPE particles in both size fractions and with small PLA/PBAT produced less CO_2 than the MP-free soil (Fig. S6-6).

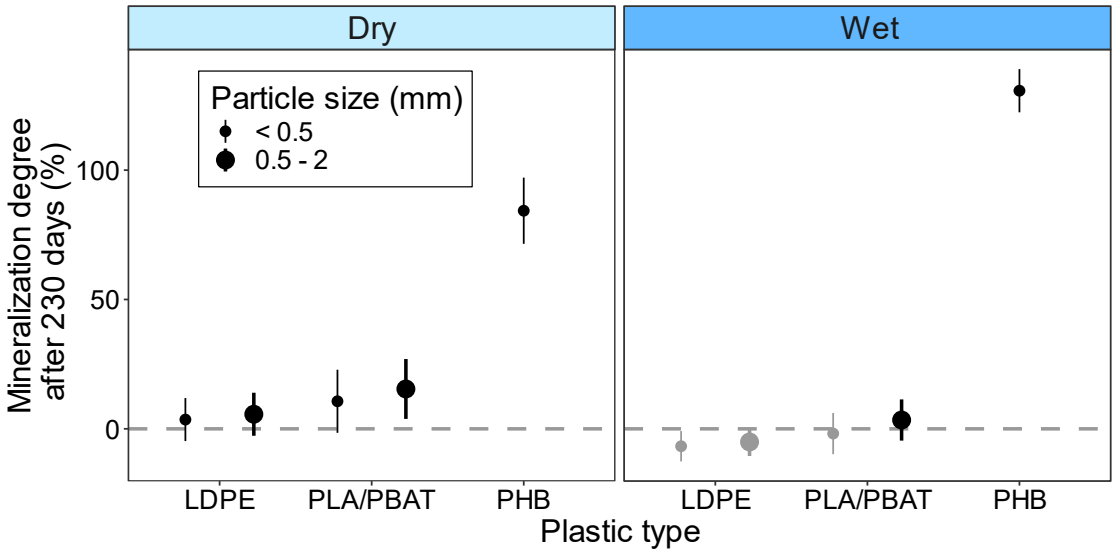

Fig. 6-1. Percentage of mineralized MP-C in relation to initially applied MP-C (mineralization degree) of small and large (< 0.5/ 0.5 – 2 mm) LDPE, PLA/PBAT, and PHB (< 0.5 mm, positive control) particles in dry and wet soil (pF = 4/ pF = 2) after 230 days. The figures show Dunnett's contrasts of cumulative CO_2 production from MP-amended compared to MP-free soil after 230 days and are expressed in percent of initially applied MP-C. Error bars show 95 % confidence intervals of the contrasts. The gray-dashed line shows 0% mineralization. Negative values (gray) are due to lower cumulative CO_2 production in MP-amended soil compared to the corresponding MP-free control soil (Fig. S6-6c).

Composition of main microbial groups and specific enzyme activities

We assessed MP effects on soil microorganisms based on PLFAs concentrations in soil, specific microbial respiration rates, and specific activities of C cycling enzymes (Fig. 6-2, Fig. S6-7). The addition of PLA/PBAT and LDPE particles to soil (1 g kg^{-1}) did not lead to effects on the composition and abundance of main microbial groups and specific enzyme activities (Fig. 6-2, Table S6-4, Fig. S6-7). However, the addition of the reference polymer PHB to soil increased PLFAs of Gram-negative (+18.6 %) and general bacteria (+10.4 %) compared to the control soil (Fig. S6-8c, Table S6-5). In dry soil, PHB addition further increased Gram-positive PLFAs and the sum of microbial PLFAs compared to the control (Table S6-6). We found that PHB addition stimulated the specific microbial respiration rate after 104 days, but this effect diminished after 230 days (Table S6-6, Fig. S6-8b). Specific enzyme activities were not affected by PHB addition to soil (Fig. S6-8a).

Regardless of MP addition, abundance and composition of the main microbial groups was different in wet compared to dry soil (*pF*, $F_{1,24}$ = 13.49 – 193.39, $p < 0.05$) and at 104 days compared to 230 days (*Timepoint*, $F_{1,24}$ = 6.61– 403.68, $p < 0.05$). In general, the microbial abundance of the main microbial groups was lower in wet compared to dry soil (Fig. S6-7,

Table S6-7, Table S6-8). This difference was greater at 230 days for general bacterial and Gram-positive PLFAs (Table S6-8). Microbial PLFAs predominantly decreased from 104 to 230 days (Fig. S6-7, Table S6-7, Table S6-9). In contrast to this, the abundance of Gram-positive PLFAs did not change significantly in dry soil from 104 to 230 days (Fig. S6-7a, Table S6-9).

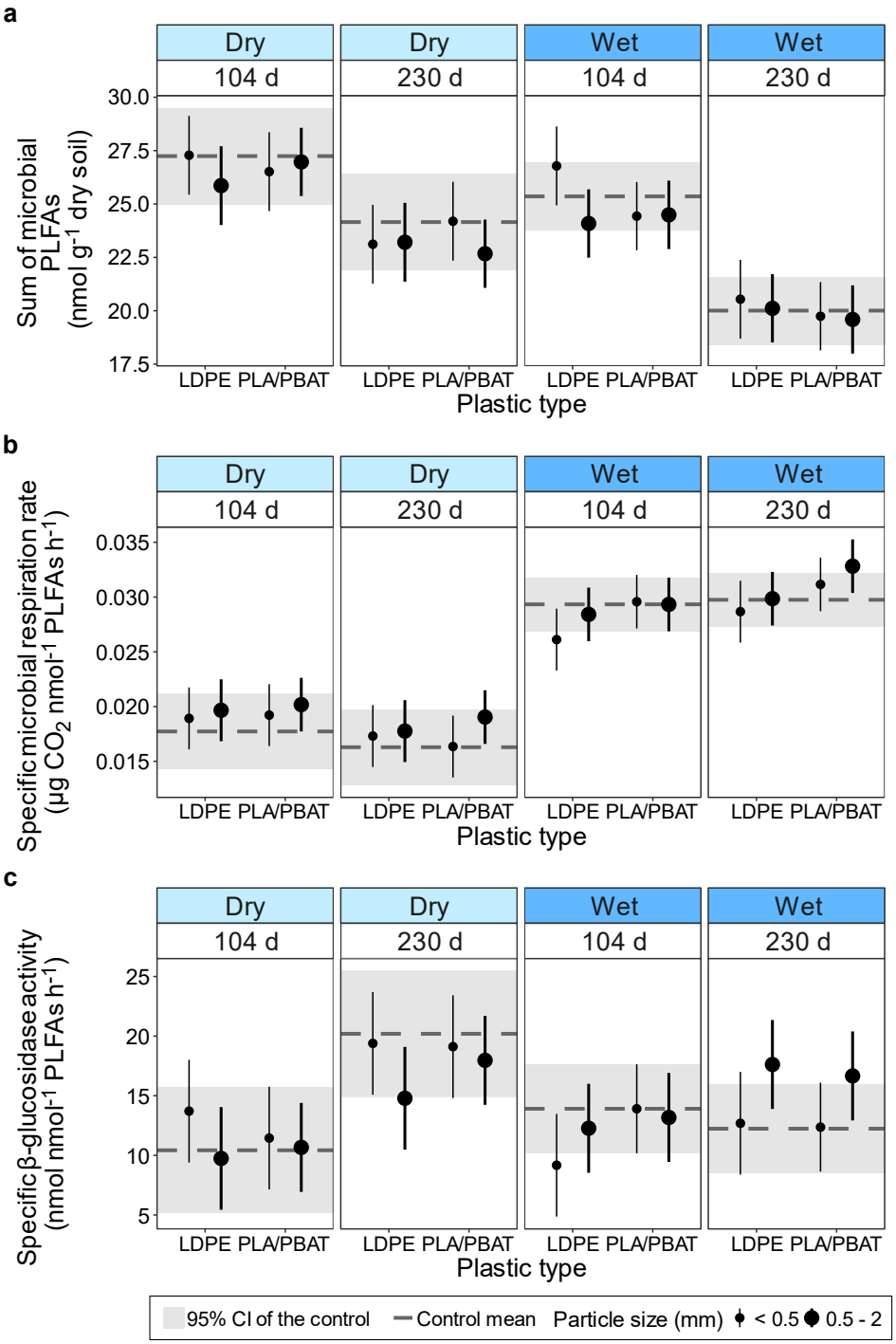

Fig. 6-2. **a** Sum of microbial PLFAs, **b** specific microbial respiration rate, and **c** specific ß-glucosidase activity in response to small and large (< 0.5 / 0.5-2 mm) LDPE and PLA/PBAT particles in dry and wet soil (pF = 4/ pF = 2) after 104 and 230 days. Specific microbial respiration rate and specific ß-glucosidase activity are both related to the sum of microbial PLFAs (nmol). Data are presented as estimated marginal means and 95 % confidence intervals (error bars) obtained from linear mixed effects models. The dashed line and the gray-colored area, respectively, display the mean and the 95% confidence intervals of the MP-free control soil.

Morphological changes on the surfaces of biodegradable MP

We used scanning electron microscopy to detect morphological surface changes of MP particles. While we did not find indications for morphological changes after incubation in soil for 230 days for LDPE particles (Fig. 6-3c and g), some PLA/PBAT particles exhibited cracks in their surface structure (Fig. 6-3e and i). These cracks in the surface of PLA/PBAT particles appeared to be rather continuous in wet soil but more sporadic in dry soil (Fig. 6-3i compared to Fig. 6-3e). In comparison, pristine MP from PLA/PBAT showed an intact and rather smooth surface (Fig. 6-3b). We did not observe microbial colonization on the surfaces of MP particles (Fig. 6-3d, f, h, and j).

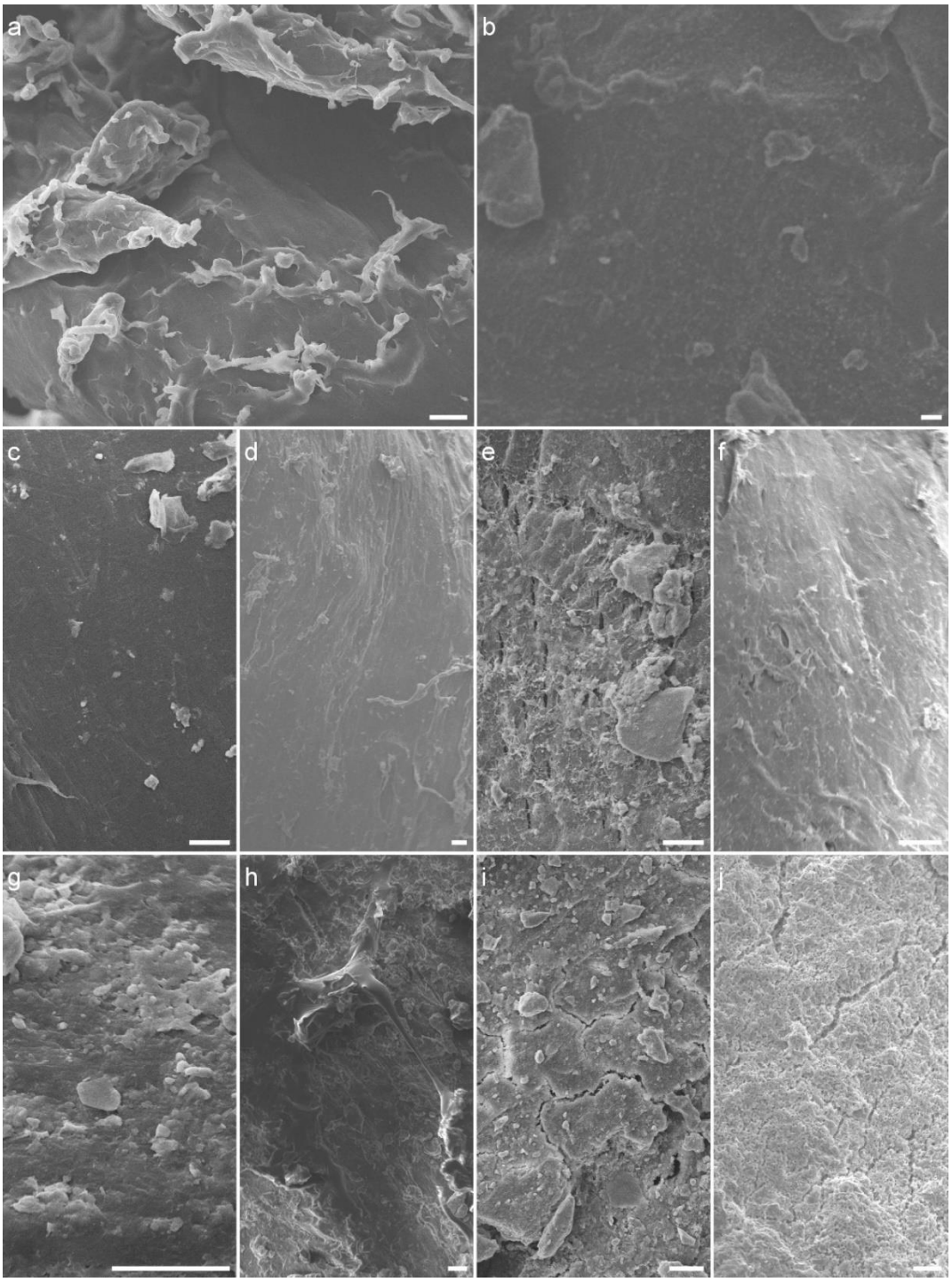

Fig. 6-3. Scanning electron microscopy images of pristine MP and extracted from soil. **a** Surface of pristine LDPE. **b** Surface of pristine PLA/PBAT. Representative images to investigate morphological changes of the surface **c** of LDPE from dry soil (pF = 4), **e** PLA/PBAT from dry soil (pF = 4), **g** LDPE from wet soil (pF = 2), **i** and PLA/PBAT from wet soil (pF = 2). Representative images to investigate biofilm formation on **d** LDPE from dry soil (pF = 4), **f** PLA/PBAT from dry soil (pF = 4), **h** LDPE from wet soil (pF = 2), **j** and PLA/PBAT from wet soil (pF = 2). All images were taken with a secondary electron detector. To investigate pristine particles and morphological changes, E0 was 15 kV and Ib was 10 pA. To investigate biofilm formation, E0 was 10 kV or Ib was 100 pA. Scale bars: 0.02 mm.

Enzyme activities on the surfaces of individual MP particles

We measured lipase and ß-glucosidase activities on the surface of individual MP particles.

Plastic type ($F_{1,11}$ = 67.16, p < 0.001) and soil moisture ($F_{1,11}$ = 29.65, p < 0.001) controlled lipase activity on the surface of MP particles. Lipase activities were significantly higher on PLA/PBAT than on LDPE particles (Table S6-10). Lipase activities on the surfaces of MP particles incubated in dry soil were higher than in wet soil (Table S6-10). In addition, the proportion of MP particles with significant lipase activity was higher for PLA/PBAT (97.8 and 93.6 % in dry and wet soil, respectively) than for LDPE (84.8 and 55.6 % in dry and wet soil, respectively) (Fig. S6-5). Surface-related median lipase activity of PLA/PBAT particles extracted from dry soil (0.28 nmol mm^{-2} MP h^{-1}) and wet soil (0.07 nmol mm^{-2} MP h^{-1}) was 9099 and 2180 times that of the adjacent soil, respectively (Fig. 6-4). For LDPE, surface-related median lipase activities of particles were enhanced by a factor of 619 (wet soil) to 1029 (dry soil) compared to adjacent soil.

The median ß-glucosidase activity of the incubated PLA/PBAT particles in dry soil (0.02 nmol mm^{-2} MP h^{-1}, 76.6 % of the particles with significant activity) was enhanced in comparison to the adjacent soil (8.32×10^{-6} nmol mm^{-2} soil h^{-1}). Only 25.5 % of PLA/PBAT particles which were extracted from wet soil and few LDPE particles which were extracted from dry (8.6 %) and wet soil (2.2 %) showed ß-glucosidase activity (Fig. 6-4, Fig. S6-5). However, individual PLA/PBAT particles incubated in wet soil as well as LDPE particles extracted from both dry and wet soil had 248 – 12,404 times higher ß-glucosidase activity than adjacent soil (Fig. 6-4).

Non-incubated MP exhibited no enzyme activity. MP particles had light brown spots (Fig. S6-9a, b, and e). However, we found high lipase activities of up to 1.77 nmol mm^{-2} MP h^{-1} on particles without discoloration (Fig. S6-9c).

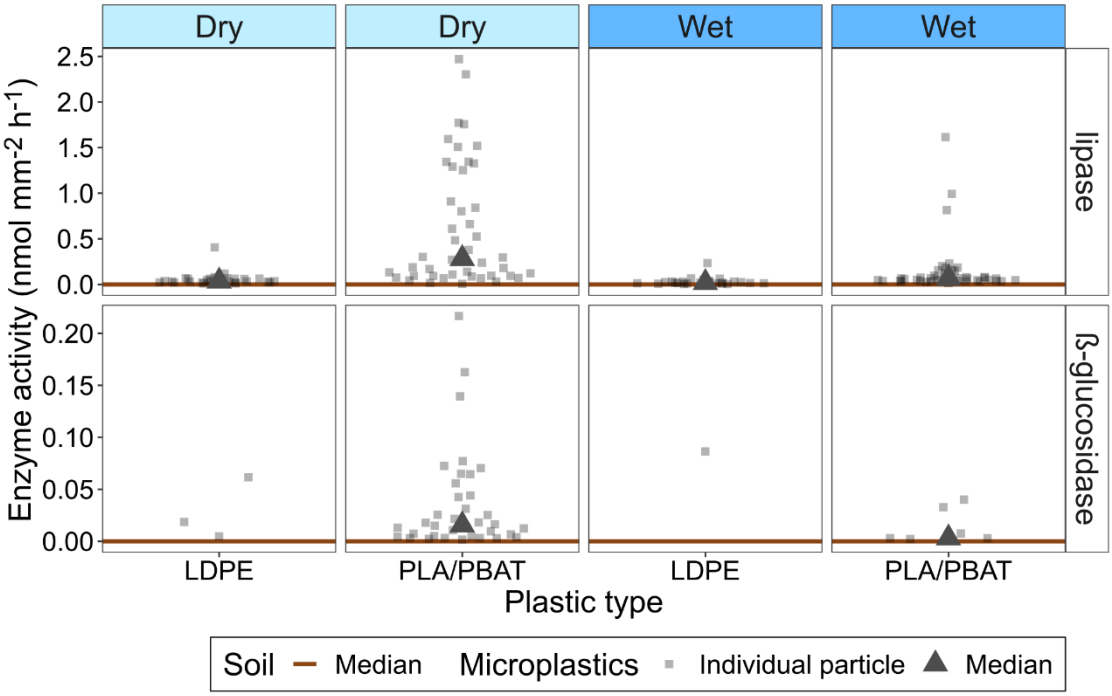

Fig. 6-4. Lipase and ß-glucosidase activities related to the surface of large MP particles (> 0.5 mm) extracted from soil as a function of plastic type (LDPE and PLA/PBAT) in dry (pF = 4) and wet soil (pF = 2). Brown lines show median enzyme activities of soil from which MP particles were extracted. Note that only one LDPE particle showed ß-glucosidase activity in wet soil; other LDPE particles did not show ß-glucosidase activity (cf. Fig. S6-5).

6.5 Discussion

Low mineralization of hydrolyzable MP particles in the soil

We investigated the biodegradability of hydrolyzable (PLA/PBAT) and non-hydrolyzable (LDPE) MP particles in soil based on MP-C mineralization. As hypothesized, no mineralization of LDPE occurred. This was expected because polymer chains of LDPE are only accessible to extracellular hydrolytic enzymes after initial chemical oxidation catalyzed by heat or UV light (Krueger et al. 2015; Restrepo-Flórez et al. 2014). Only large PLA/PBAT particles incubated in dry soil were significantly mineralized. Their mineralization was low (15.4 % within 230 days) in comparison to the reference polymer PHB (84.3 %) under the same conditions. While biodegradation studies on particles are lacking, a few studies have investigated the mineralization of PLA/PBAT films (Freitas et al. 2017; Palsikowski et al. 2018). In these studies, similar levels of PLA/PBAT-C mineralization were observed; 10 % within 180 days for PLA/PBAT (75/25 % w/w) and 18 % within 126 days for PLA/PBAT (45/55 % w/w). The relatively low mineralization of the PLA/PBAT polymer in our study is most likely related to

the low PBAT content (15 %). We suggest that PBAT was selectively mineralized since in soil PBAT is typically more biodegradable than PLA (Palsikowski et al., 2018; Weng et al., 2013). The observed lack of PLA/PBAT mineralization in wet soil (pF = 2) might be explained by lower abundances of PLA/PBAT degrading microorganisms due to overall fewer microorganisms in wet soil (Fig. 6-2a, Table S6-7, Table S6-8, and Fig. S6-7).

The overestimation of PHB mineralization in wet soil (130.7 %, Fig. 6-1) is likely due to the sudden increase in CO_2 production rates in the first 2 weeks of the experiment (Fig. S6-6a). Possibly, the PHB addition in wet soil (pF 2) triggered a stress response in microorganisms, resulting in increased basal respiration compared to the MP-free control soil.

Soil microorganisms – unaffected by MP?

While we hypothesized that MP would affect soil microorganisms and their activity, the addition of both LDPE- and PLA/PBAT-MP to soil (1 g MP kg^{-1} dry soil ≙ 0.1 % w/w) had no effect on abundance and composition of the main soil microbial groups, specific respiration rates, and specific enzyme activities in our study. Interestingly, the reference polymer PHB stimulated the growth of Gram-negative bacteria, which most likely used PHB as an energy and C source (Meereboer et al. 2020). However, we found no increased specific lipase activities (Fig. S6-8) that could catalyze the biodegradation of PHB (Meereboer et al. 2020). Presumably, the hydrolyzation intensity and lipase activity were highest at the beginning of the incubation, when most PHB mineralization occurred (up to 100 days, Fig. S6-6a and b). After 104 and 230 days, lipase activities may have stabilized to background levels. Yet, also other enzymes, e.g., PHB depolymerase, may have catalyzed the degradation of PHB (Tokiwa and Calabia 2007).

Effects of MP from PLA/PBAT blends on the composition of main microbial groups and enzyme activities in soil have not yet been studied, but there is evidence that PLA/PBAT mulch films (20/80% w/w) influence soil microbial community composition (Min Zhang et al. 2019). The authors found a relatively higher abundance of potentially PLA/PBAT degrading bacterial species (*Sphingomonas, Bacillus, Streptomyces*) in the soil adjacent to the films. In contrast to this, pure PBAT films ($2 \times 2 \times 0.1$ cm^3) in soil were found to promote the growth of Ascomycota fungi adjacent to the film surfaces with impacts on the fungal composition in the bulk soil compared to the plastic-free control soil (Muroi et al. 2016). The authors proposed that the weight loss of PBAT films they observed after 7 months in soil was due to degradation processes (hydrolysis and mineralization) by these specific fungi since they can produce cutinase-like enzymes that are involved in the degradation of PBAT (Muroi et al. 2016). In contrast to these findings, based on our PLFAs analyses, we could not identify increases in the

abundances of the main microbial groups (fungi, Gram-negative, and Gram-positive bacteria) in MP-amended compared to MP-free soil (Fig. S6-7). In accordance with our results, Chen et al. (2020) found no significant effects of PLA-MP (20 – 50 µm, 2 % w/w) on bacterial community composition or on soil enzyme activity. The authors attributed the absence of effects on soil microbial processes to the persistence of PLA in soil. The low biodegradability of PLA/PBAT-MP in our study could explain why there were no MP-induced effects on the composition of the main microbial groups and specific enzyme activities. However, the studies cited above are not directly comparable to our study because we used different shapes (compared to Min Zhang et al. 2019 and Muroi et al. 2016), sizes (compared to Chen et al. 2020), chemical compositions, and soil types (compared to all cited studies). Therefore, we propose to systematically investigate the potential impacts of PLA/PBAT in soil by considering these factors to identify the most important ones.

Given the persistence and resistance of LDPE to microbial attack (Krueger et al. 2015), it is unlikely that LDPE promotes the growth of specific microbial taxa that would utilize LDPE-C, and even less likely if readily available C sources are present in the soil, as is usually the case in agricultural soils (Ng et al. 2018). However, Souza Machado et al. (2019) provided evidence for alterations in the soil microbial habitat due to MP. High-density-polyethylene fragments (2 % w/w, 643 µm average size) decreased soil bulk density and increased evapotranspiration, which was associated with stimulation in microbial metabolic activity compared to the control soil. Such habitat alterations can affect bacterial community richness and diversity in soil. For example, Fei et al. (2020) reported that the addition of LDPE-MP (mean of 678 µm, 1 – 5 % w/w) caused significant increases in the relative abundance of specific bacteria families in comparison to the control, (e.g., Pseudomonoadaceae, Burkholderiaceae), while restricting the growth of others (e.g., Xanthobacteraceae, Caulobacteraceae). In the same study, these changes in microbial community composition were associated with enhanced urease and acid phosphatase activities but reductions in fluorescein diacetate hydrolase activity when compared to the control soil.

Nevertheless, consistent with our findings, Blöcker et al. (2020) did not observe significant effects of LDPE-MP (200 – 630 µm, 1 % w/w) on the composition of the main microbial groups in soil and microbial respiration, compared to MP-free soil. However, they found a decrease in microbial biomass C and N. In contrast, Wiedner and Polifka (2020) observed an increase (non-significant, however) in the sum of microbial PLFAs in response to LDPE-MP (< 100 µm, 1 % w/w). Blöcker et al. (2020) suggested limited availability of LDPE-C in the vicinity of the MP and sorption of cations to the negatively charged MP surfaces, thus restricting the accessibility

of essential cations for the microorganisms' metabolism, explained their observed decrease in microbial biomass. In contrast, Wiedner and Polifka (2020) proposed that LDPE could favor the formation of microbial habitats and by this promote the growth of microorganisms in the soil.

In addition to polymer- and soil-specific properties that may control LDPE-MP effects in the soil, it is likely that the relatively low MP concentration used in our study, although well above typical current MP loads in agricultural soils (Büks and Kaupenjohann 2020), was below a critical level compared to the studies cited above (0.1% in our study compared to 1 – 5% w/w), so no such physicochemical interactions of MP with soil occurred in our study. However, artificial laboratory conditions were used in our study and in the studies mentioned above. In contrast, under field conditions, MP may age on the soil surface due to the influence of UV light, which in turn may affect the behavior of MP in soil. Therefore, field experiments with MP are essential to estimate such influences on MP behavior in soil and to verify laboratory findings.

The plastisphere – a specific microbial habitat in soil?

Consistent with our third hypothesis that PLA/PBAT surfaces would show morphological changes, PLA/PBAT particles extracted from dry and wet soils exhibited cracks in their surface structure (Fig. 6-3e and i). In contrast, the surface morphology of LDPE did not show any visual changes after incubation in soil compared to the pristine, non-incubated particles that had smooth surfaces (Fig. 6-3a, c, and g). In addition, lipase activities of individual PLA/PBAT particles were enhanced compared to LDPE particles, but contrary to our expectation, higher in dry than in wet soil. Lipase activities on PLA/PBAT surfaces were higher than those in MP-amended soil (up to 9099 times that of MP-amended soil, Fig. 6-4). ß-Glucosidase activity also increased on PLA/PBAT particles after incubation in dry soil.

Morphological changes paired with lipase activities on the surfaces of individual PLA/PBAT particles indicate hydrolysis of PLA/PBAT, thus initiation of PLA/PBAT biodegradation (Lamparelli et al. 2021; Zumstein et al. 2018). In accord with higher lipase activities on the surface of PLA/PBAT particles in dry compared to wet soils, we found mineralization of these particles in dry soil but none for those incubated in wet soil. Although we observed no colonization via scanning electron microscopy (Fig. 6-3f and j), we assume that lipase activity was triggered by the formation of a specific plastisphere microbiome adjacent to PLA/PBAT-MP surfaces in soil that consisted of lipase-producing microorganisms. Our assumption is supported by Rüthi et al. (2020) who found evidence for specific plastisphere microbiomes of

PLA, PBAT and PE (film plastic pieces of 4 × 4 cm^2) in alpine and arctic soils. They identified Saccharibacteria as key members of the plastisphere microbiome of PLA, some of which can produce lipases and other extracellular enzymes that can catalyze PLA degradation. As our PLA/PBAT blend had a high proportion of PLA (85 %), an enrichment of such specific taxa could explain the high lipase activities on the surface of PLA/PBAT particles. Since Saccharibacteria were identified as having Gram-positive cell structures (Hugenholtz et al. 2001) and we observed a higher abundance of Gram-positive PLFAs in dry than in wet soil after 230 days (Fig. S6-7, Table S6-8), we suggest that there may have been more lipase-producing Gram-positive bacteria such as Saccharibacteria in dry compared to wet soil. This could also explain higher lipase activities on PLA/PBAT particles and the higher mineralization of PLA/PBAT in the dry compared to the wet soil.

Given the lipase activities on PLA/PBAT surfaces, hydrolyzing enzymes were most likely not the limiting factor in our study, in contrast to Zumstein et al. (2017), who found enzymatic hydrolysis to be the controlling process in the biodegradation of PBAT. Therefore, we suggest that either the polymer structure impeded the steric accessibility of hydrolytic enzymes due to its crystallinity (Meereboer et al. 2020; Palsikowski et al. 2018) or that soil microorganisms capable of utilizing the hydrolysis products as energy and C source were in low abundance (Meyer-Cifuentes et al. 2020).

The higher lipase activity of PLA/PBAT-MP in dry compared to wet soil could also be due to the hydrophilicity of PLA/PBAT surfaces, which was found to increase due to incubation in soil (Lamparelli et al. 2021; Osman et al. 2014). PLA/PBAT surfaces may have acted as micro-hydrological niches for soil microorganisms in dry soil, analogous to mucilage and extracellular polymeric substances (Benard et al. 2019). Accordingly, PLA/PBAT-particles in dry soil may provide wetter surfaces compared to adjacent soil particles and thus be more attractive for microorganisms. As a result, MP particles could have become microbial hotspots. In wet soil, however, the difference from MP to adjacent soil particles might not have been as pronounced as in dry soil.

The cracks observed on the surfaces of PLA/PBAT particles after incubation in soil may also have resulted from abiotic hydrolysis (Husárová et al. 2014; Yang et al. 2021). This could have paved the way for microbial action on the MP surface. Brown spots identified by light microscopy (Fig. S6-9) likely represent clay minerals or iron oxides as were also observed on MP sediment samples (Corcoran et al. 2015; Zhou et al. 2016). Clay minerals adhered to MP, as biogeochemically reactive surfaces, offer microhabitats for microorganisms (Boeddinghaus et al. 2021; Kandeler et al. 2019) and could promote microbial processes on the MP surface.

Thus, the interaction of plastisphere-mineralosphere and soil water could control microbial processes on the MP surface.

6.6 Conclusion

We studied the biodegradability of hydrolyzable MP (PLA/PBAT) and non-hydrolyzable MP (LDPE) and their potential effects on microbial abundance and composition of the main soil microbial groups (fungi, Gram-negative, and Gram-positive bacteria) as well as on microbial processes (C cycling) in an agricultural soil. In addition, we examined morphological changes and specific enzyme activities (lipases) on the surfaces of MP particles.

We detected low mineralization of PLA/PBAT-MP under rather dry conditions, which was most likely initialized by hydrolytic action of lipases on the surface of PLA/PBAT. The observation of cracks in the surface structure of these PLA/PBAT particles is likely the result of these hydrolytic processes but can also be related to the influence of soil water. Lipase activities on the PLA/PBAT surfaces were higher in comparison to the adjacent MP-amended soil, suggesting that these may provide microbial habitats for specific microorganisms (lipase-producing microorganisms) in the proximity of PLA/PBAT particles. This supports the formation of a plastisphere in the soil in our study, which was controlled by the plastic type and soil moisture. However, the influence of the plastisphere was probably locally restricted, as we found no effects of MP on microbial abundance and composition of the main microbial groups and microbial processes in MP-amended soil.

Our results suggest that hydrolyzable MP can also persist in soil. Through the ongoing application of organic fertilizers from bio-waste processing plants, MP will accumulate, and their concentrations will increase in soil. With rising concentrations, the negative effects of MP on soil microorganisms and their functions cannot be ruled out. To estimate this risk adequately, systematic long-term studies that consider disintegration, fragmentation, and the transport of MP in agricultural soils are imperative. Upcoming studies should focus on the polymer-specific plastisphere in different soils to obtain more information about the impact of this new anthropogenic microbial habitat on microbially-driven soil processes.

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6.8 Author contribution

Conceptualization: Lion Schöpfer, Sven Marhan, Ellen Kandeler, Holger Pagel; investigation: Lion Schöpfer, Uwe Schnepf; formal analysis: Lion Schöpfer; Uwe Schnepf; visualization: Lion Schöpfer; Uwe Schnepf; data curation: Lion Schöpfer; Uwe Schnepf; writing –original draft: Lion Schöpfer; writing – reviewing and editing: Lion Schöpfer, Uwe Schnepf, Sven Marhan, Franz Brümmer, Ellen Kandeler, Holger Pagel; funding acquisition: Franz Brümmer, Ellen Kandeler, Holger Pagel.

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6.10 Data availability

The datasets generated and/or analyzed during the current study are available on Mendeley Data repository, https://doi.org/10.17632/22jwmgvjcr.3.

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6.12 Supplementary information

Supplementary information is available at https://link.springer.com/article/10.1007/s00374-022-01638-9#Sec23

1 Particle size and shape distributions

Particle size and shape distributions of MP were obtained by static image analysis based on international standards (International Organization for Standardization 2014). Stitches were taken with a digital 3D-microscope (VHX-7000 & VHX-S650E, KEYENCE CORPORATION, Osaka, Japan) using either ZS20 or ZS200 objective (size fraction < 0.5 mm: scale = 870 px mm^{-1}, total size: 17,243 × 18,834 – 17,304 × 18,887 px; size fraction 0.5 – 2 mm: scale = 280 px mm^{-1}, total image size: 6540 × 6650 px; and PHB: scale = 3610 px mm^{-1}, total image size: 17,300 × 18,890 px).

Images were processed with a customized macro in Fiji 1.52p (Schindelin et al. 2012; Schneider et al. 2012). No pre- or post-processing was done. Automatic thresholding was performed using the Otsu algorithm (Otsu 1979). Particles were then measured with the "Particles8_v2.19" function of the Morphology plugin by Landini (2008). In total, 16,072 MP particles were measured for LDPE < 0.5 mm; 5451 for LDPE 0.5 – 2 mm; 10,004 for PLA/PBAT < 0.5 mm; 697 for PLA/PBAT 0.5 – 2 mm; and 8370 for PHB.

According to Shekunov et al. (2007), data entries with a Feret's diameter ≤ 3 µm were completely removed due to the measurement range of light microscopes, and MP smaller or

larger than the respective mesh size of the sieves were also not considered in the subsequent analysis (for details, refer to "Microplastics preparation and characteristics" section, Materials and methods; LDPE < 0.5 mm: 19.94 %, PLA/PBAT < 0.5 mm: 36.13 %, LDPE 0.5 – 2 mm: 96.72 %, PLA/PBAT 0.5 – 2 mm: 68.72 %, PHB: 32.01 %). The width of particle size distributions was categorized as defined by Merkus (2009).

Shape descriptors (sphericity, elongation, and solidity) were calculated as described in the literature (Crompton 2005; Riley 1941). If data entries exceeded the maximum values of the theoretical *Sphericity versus Elongation* model proposed by Kröner and Doménech Carbó (2013), they were also removed (LDPE < 0.5 mm: 0.23%, LDPE 0.5 – 2 mm: 0 %, PLA/PBAT < 0.5 mm: 0.55 %, PLA/PBAT 0.5 – 2 mm: 0 %, PHB: 0 %, Fig. S6-2)

Supplementary figures

Table S6-1. Summary statistics for particle size and shape of the different plastic types and size fractions used in this study. D_{50} is the median of the sample, IQR its interquartile range, D_{90} its 90th percentile, D_{10} its 10th percentile, and SD means standard deviation. The width of particle size distributions was determined according to Merkus (2009). Proportions of fibers were calculated based on the criterion proposed by Cole (2016).

			Size								Spher-icity		Elong-ation		Soli-dity		Fibers
Plastic type	**Fraction**	**Particle number**	**Average (mm)**	**SD (mm)**	**D_{50} (mm)**	**IQR (mm)**	**D_{10} (mm)**	**D_{90} (mm)**	**D_{90}/D_{10}**	**Distri-bution width**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Pro-portion (%)**
LDPE	0.5 - 2 mm	179	0.907	0.361	0.806	0.459	0.550	1.511	2.75	medium	0.15	0.12	0.43	0.17	0.59	0.14	6
	< 0.5 mm	12,839	0.074	0.079	0.051	0.092	0.005	0.174	33.80	very broad	0.29	0.18	0.42	0.18	0.79	0.15	9
PHB		5,691	0.014	0.018	0.008	0.010	0.004	0.028	7.20	broad	0.40	0.16	0.28	0.14	0.84	0.08	0
PLA/PBAT	0.5 – 2 mm	214	0.866	0.252	0.813	0.325	0.587	1.200	2.04	medium	0.47	0.17	0.25	0.13	0.87	0.09	1
	< 0.5 mm	6,355	0.073	0.094	0.024	0.106	0.004	0.210	50.78	very broad	0.32	0.17	0.41	0.19	0.82	0.15	10

Table S6-2. Experimental design. The second header line shows names of the factors as used in the data evaluation. Dry and wet soil are here presented with their pF values (pF = 4 and pF = 2, respectively). The particle size fractions of < 0.5 and 0.5 – 2 mm were considered as small and large MP, respectively.

Incubation days	**Soil moisture (pF)**	**Control / Treatment**	**Plastic type**	**Particle size (mm)**	**n**
Timepoint	***pF***	***ConVSTrt***	***PlasticType***	***ParticleSize***	
104 d	4	Con			2
		Trt	LDPE	< 0.5	3
				0.5 – 2	3
			PLA/PBAT	< 0.5	3
				0.5 – 2	4
	2	Con			4
		Trt	LDPE	< 0.5	3
				0.5 – 2	4
			PLA/PBAT	< 0.5	4
				0.5 – 2	4
230 d	4	Con			2
		Trt	LDPE	< 0.5	3
				0.5 – 2	3
			PLA/PBAT	< 0.5	3
				0.5 – 2	4
	2	Con			4
		Trt	LDPE	< 0.5	3
				0.5 – 2	4
			PLA/PBAT	< 0.5	4
				0.5 – 2	4

Table S6-3. Dunnett's contrasts for cumulative CO_2 production over 230 days of MP-amended soil and MP-free control soil and percentage of mineralized MP-C in relation to initially applied MP-C (mineralization degree) of small and large (< 0.5 / 0.5 – 2 mm) LDPE, PLA/PBAT and PHB (< 0.5 mm, positive control) particles in dry and wet soil (pF = 4 / pF = 2), respectively.

Soil moisture level	**Plastic type**	**Particle size (mm)**	**Difference in cumulative CO_2 production to the control with [lower; upper 95% CI] (mg CO_2 g^{-1} dry soil)**	**Mineralization of initially added MP-C with [lower; upper 95% CI] (%)**	***df***	**Dunnett's *t***	***p***
Dry	PHB	< 0.5	1.72 [1.46; 1.98]	84.3 [71.5; 97.1]	12	19.28	< 0.001
	LDPE	< 0.5	0.11 [-0.15; 0.37]	3.6 [-4.7; 11.9]	12	1.26	0.595
		0.5 – 2	0.18 [-0.08; 0.44]	5.6 [-2.7; 14.0]	12	1.98	0.238
	PLA/PBAT	< 0.5	0.23 [-0.03; 0.49]	10.7 [-1.5; 22.8]	12	2.55	0.095
		0.5 – 2	0.33 [0.08; 0.58]	15.4 [3.8; 27.0]	12	3.90	0.009

Soil moisture level	Plastic type	Particle size (mm)	Difference in cumulative CO_2 production to the control with [lower; upper 95% CI] ($mg\ CO_2\ g^{-1}$ dry soil)	Mineralization of initially added MP-C with [lower; upper 95% CI] (%)	*df*	Dunnett's *t*	*p*
Wet	PHB	< 0.5	2.66 [2.49; 2.83]	130.7 [122.3; 139.1]	17	43.76	< 0.001
	LDPE	< 0.5	-0.21 [-0.40; -0.03]	-6.7 [-12.6; -0.9]	17	-3.21	0.022
		0.5 – 2	-0.16 [-0.33; 0.01]	-5.1 [-10.5; 0.4]	17	-2.62	0.071
	PLA/PBAT	< 0.5	-0.04 [-0.21; 0.13]	-1.8 [-9.8; 6.1]	17	-0.64	0.909
		0.5 – 2	0.07 [-0.10; 0.24]	3.4 [-4.6; 11.4]	17	1.20	0.626

Table S6-4. ANOVAs for PLFAs, specific microbial respiration rates, and specific enzyme activities with Kenward-Roger approximation of degrees of freedom (Kenward and Roger 1997). Sum of squares (sumsq), mean sum of squares (meansq), numerator degrees of freedom (*df num*), denominator degrees of freedom (*df den*), *F*-statistic (*F*), *p*-value (*p*).

Variable	Term	sumsq	meansq	*df num*	*df den*	*F*	*p*
General bacterial PLFAs	Timepoint	3.2e+00	3.2e+00	1	24	129.33	< 0.001
	pF	1.25e+00	1.25e+00	1	24	50.58	< 0.001
	ConVSTrt	1.56e-02	1.56e-02	1	24	0.63	0.435
	pF:ConVSTrt	1.7e-03	1.7e-03	1	24	0.07	0.796
	Timepoint:pF	1.32e-01	1.32e-01	1	24	5.34	0.03
	Timepoint:ConVSTrt	4.18e-03	4.18e-03	1	24	0.17	0.685
	pF:ConVSTrt:PlasticType	7.61e-02	3.81e-02	2	24	1.54	0.235
	pF:ConVSTrt:ParticleSize	1.13e-01	5.63e-02	2	24	2.28	0.125
	Timepoint:pF:ConVSTrt	2.98e-03	2.98e-03	1	24	0.12	0.732
	pF:ConVSTrt:PlasticType:ParticleSize	6.24e-02	3.12e-02	2	24	1.26	0.302
	Timepoint:pF:ConVSTrt:PlasticType	3.39e-03	1.7e-03	2	24	0.07	0.934
	Timepoint:pF:ConVSTrt:ParticleSize	4.57e-02	2.29e-02	2	24	0.92	0.411
	Timepoint:pF:ConVSTrt:PlasticType:ParticleSize	9.73e-02	4.87e-02	2	24	1.97	0.162
Sum of bacterial PLFAs	Timepoint	3.94e+01	3.94e+01	1	24	36.12	< 0.001
	pF	3.95e+01	3.95e+01	1	24	36.19	< 0.001
	ConVSTrt	3.35e-01	3.35e-01	1	24	0.31	0.585
	pF:ConVSTrt	1.88e-01	1.88e-01	1	24	0.17	0.682
	Timepoint:pF	5.96e+00	5.96e+00	1	24	5.46	0.028
	Timepoint:ConVSTrt	4.43e-02	4.43e-02	1	24	0.04	0.842
	pF:ConVSTrt:PlasticType	1.62e+00	8.08e-01	2	24	0.74	0.488

Variable	Term	sumsq	meansq	*df num*	*df den*	*F*	*p*
	pF:ConVSTrt:ParticleSize	2.69e+00	1.34e+00	2	24	1.23	0.31
	Timepoint:pF:ConVSTrt	9.23e-02	9.23e-02	1	24	0.08	0.774
	pF:ConVSTrt:PlasticType:ParticleSize	2.08e+00	1.04e+00	2	24	0.95	0.399
	Timepoint:pF:ConVSTrt:PlasticType	2.21e-01	1.1e-01	2	24	0.10	0.904
	Timepoint:pF:ConVSTrt:ParticleSize	8.01e-01	4.01e-01	2	24	0.37	0.697
	Timepoint:pF:ConVSTrt:PlasticType:ParticleSize	3.13e+00	1.57e+00	2	24	1.43	0.258
Specific ß-glucosidase activity	Timepoint	2.76e+02	2.76e+02	1	24	26.38	< 0.001
	pF	1.36e+01	1.36e+01	1	24	1.30	0.266
	ConVSTrt	8.17e-02	8.17e-02	1	24	0.01	0.930
	pF:ConVSTrt	2.36e+00	2.36e+00	1	24	0.23	0.639
	Timepoint:pF	1.13e+02	1.13e+02	1	24	10.79	0.003
	Timepoint:ConVSTrt	6.48e+00	6.48e+00	1	24	0.62	0.439
	pF:ConVSTrt:PlasticType	3.38e+00	1.69e+00	2	24	0.16	0.852
	pF:ConVSTrt:ParticleSize	6.12e+01	3.06e+01	2	24	2.92	0.073
	Timepoint:pF:ConVSTrt	3.23e+01	3.23e+01	1	24	3.09	0.092
	pF:ConVSTrt:PlasticType:ParticleSize	1.65e+01	8.26e+00	2	24	0.79	0.465
	Timepoint:pF:ConVSTrt:PlasticType	3.12e+01	1.56e+01	2	24	1.49	0.245
	Timepoint:pF:ConVSTrt:ParticleSize	2.38e+01	1.19e+01	2	24	1.14	0.337
	Timepoint:pF:ConVSTrt:PlasticType:ParticleSize	4.74e+00	2.37e+00	2	24	0.23	0.799
Specific microbial respiration rate	Timepoint	1.14e-06	1.14e-06	1	24	0.19	0.665
	pF	2.1e-03	2.1e-03	1	24	354.34	< 0.001
	ConVSTrt	3.91e-06	3.91e-06	1	24	0.66	0.425
	pF:ConVSTrt	5.32e-06	5.32e-06	1	24	0.90	0.353
	Timepoint:pF	5.53e-05	5.53e-05	1	24	9.33	0.005
	Timepoint:ConVSTrt	2.67e-06	2.67e-06	1	24	0.45	0.508
	pF:ConVSTrt:PlasticType	4.17e-05	2.09e-05	2	24	3.52	0.046
	pF:ConVSTrt:ParticleSize	2.05e-05	1.03e-05	2	24	1.73	0.199
	Timepoint:pF:ConVSTrt	2.74e-06	2.74e-06	1	24	0.46	0.503
	pF:ConVSTrt:PlasticType:ParticleSize	4.38e-06	2.19e-06	2	24	0.37	0.695
	Timepoint:pF:ConVSTrt:PlasticType	7.52e-07	3.76e-07	2	24	0.06	0.939

Variable	Term	sumsq	meansq	*df num*	*df den*	*F*	*p*
	Timepoint:pF:ConVSTrt:ParticleSize	1.5e-06	7.5e-07	2	24	0.13	0.882
	Timepoint:pF:ConVSTrt:PlasticType:ParticleSize	5.88e-06	2.94e-06	2	24	0.50	0.615
Fungal PLFAs	Timepoint	5.3e-01	5.3e-01	1	24	143.13	< 0.001
	pF	3.47e-01	3.47e-01	1	24	93.63	< 0.001
	ConVSTrt	2.53e-03	2.53e-03	1	24	0.68	0.417
	pF:ConVSTrt	3.1e-03	3.1e-03	1	24	0.84	0.369
	Timepoint:pF	3.29e-04	3.29e-04	1	24	0.09	0.768
	Timepoint:ConVSTrt	2.87e-03	2.87e-03	1	24	0.77	0.388
	pF:ConVSTrt:PlasticType	6.72e-03	3.36e-03	2	24	0.91	0.417
	pF:ConVSTrt:ParticleSize	9.19e-05	4.6e-05	2	24	0.01	0.988
	Timepoint:pF:ConVSTrt	2.79e-03	2.79e-03	1	24	0.75	0.394
	pF:ConVSTrt:PlasticType:ParticleSize	6.04e-03	3.02e-03	2	24	0.81	0.455
	Timepoint:pF:ConVSTrt:PlasticType	6.37e-03	3.18e-03	2	24	0.86	0.436
	Timepoint:pF:ConVSTrt:ParticleSize	2.87e-03	1.44e-03	2	24	0.39	0.683
	Timepoint:pF:ConVSTrt:PlasticType:ParticleSize	1.23e-03	6.14e-04	2	24	0.17	0.848
Ratio of fungal to bacterial PLFAs	Timepoint	1.2e-03	1.2e-03	1	24	30.47	< 0.001
	pF	5.31e-04	5.31e-04	1	24	13.49	0.001
	ConVSTrt	1.69e-06	1.69e-06	1	24	0.04	0.838
	pF:ConVSTrt	9.54e-06	9.54e-06	1	24	0.24	0.627
	Timepoint:pF	3.97e-05	3.97e-05	1	24	1.01	0.325
	Timepoint:ConVSTrt	1.25e-05	1.25e-05	1	24	0.32	0.578
	pF:ConVSTrt:PlasticType	5.95e-05	2.98e-05	2	24	0.76	0.48
	pF:ConVSTrt:ParticleSize	3.58e-05	1.79e-05	2	24	0.45	0.64
	Timepoint:pF:ConVSTrt	1.16e-05	1.16e-05	1	24	0.29	0.592
	pF:ConVSTrt:PlasticType:ParticleSize	3.87e-05	1.93e-05	2	24	0.49	0.618
	Timepoint:pF:ConVSTrt:PlasticType	5.56e-05	2.78e-05	2	24	0.71	0.503
	Timepoint:pF:ConVSTrt:ParticleSize	1.61e-05	8.04e-06	2	24	0.20	0.817
	Timepoint:pF:ConVSTrt:PlasticType:ParticleSize	2.48e-05	1.24e-05	2	24	0.32	0.733
Gram-negative PLFAs	Timepoint	5.09e+00	5.09e+00	1	24	403.68	< 0.001
	pF	2.44e+00	2.44e+00	1	24	193.39	< 0.001

Variable	**Term**	**sumsq**	**meansq**	***df num***	***df den***	***F***	***p***
	ConVSTrt	9.08e-04	9.08e-04	1	24	0.07	0.791
	pF:ConVSTrt	6.89e-04	6.89e-04	1	24	0.05	0.817
	Timepoint:pF	3.09e-02	3.09e-02	1	24	2.45	0.131
	Timepoint:ConVSTrt	6.52e-04	6.52e-04	1	24	0.05	0.822
	pF:ConVSTrt:PlasticType	1.79e-02	8.94e-03	2	24	0.71	0.502
	pF:ConVSTrt:ParticleSize	1.59e-02	7.93e-03	2	24	0.63	0.542
	Timepoint:pF:ConVSTrt	2.89e-03	2.89e-03	1	24	0.23	0.637
	pF:ConVSTrt:PlasticType:ParticleSize	4.46e-03	2.23e-03	2	24	0.18	0.839
	Timepoint:pF:ConVSTrt:PlasticType	1.46e-02	7.28e-03	2	24	0.58	0.569
	Timepoint:pF:ConVSTrt:ParticleSize	5.37e-03	2.68e-03	2	24	0.21	0.81
	Timepoint:pF:ConVSTrt:PlasticType:ParticleSize	1.52e-02	7.59e-03	2	24	0.60	0.556
Gram-positive PLFAs	Timepoint	5e+00	5e+00	1	24	6.61	0.017
	pF	1.3e+01	1.3e+01	1	24	17.21	< 0.001
	ConVSTrt	1.79e-01	1.79e-01	1	24	0.24	0.631
	pF:ConVSTrt	1.34e-01	1.34e-01	1	24	0.18	0.678
	Timepoint:pF	3.62e+00	3.62e+00	1	24	4.79	0.039
	Timepoint:ConVSTrt	1.45e-02	1.45e-02	1	24	0.02	0.891
	pF:ConVSTrt:PlasticType	7.52e-01	3.76e-01	2	24	0.50	0.614
	pF:ConVSTrt:ParticleSize	1.46e+00	7.3e-01	2	24	0.97	0.395
	Timepoint:pF:ConVSTrt	9.17e-02	9.17e-02	1	24	0.12	0.731
	pF:ConVSTrt:PlasticType:ParticleSize	1.44e+00	7.21e-01	2	24	0.95	0.399
	Timepoint:pF:ConVSTrt:PlasticType	3.56e-01	1.78e-01	2	24	0.24	0.792
	Timepoint:pF:ConVSTrt:ParticleSize	3.76e-01	1.88e-01	2	24	0.25	0.782
	Timepoint:pF:ConVSTrt:PlasticType:ParticleSize	1.81e+00	9.07e-01	2	24	1.20	0.318
Ratio of Gram-positive to Gram-negative PLFAs	Timepoint	2.06e+01	2.06e+01	1	24	105.80	< 0.001
	pF	3.14e+00	3.14e+00	1	24	16.13	< 0.001
	ConVSTrt	1.21e-02	1.21e-02	1	24	0.06	0.805
	pF:ConVSTrt	2.34e-02	2.34e-02	1	24	0.12	0.732
	Timepoint:pF	2.69e-02	2.69e-02	1	24	0.14	0.714
	Timepoint:ConVSTrt	8.74e-04	8.74e-04	1	24	0.00	0.947

Variable	Term	sumsq	meansq	*df num*	*df den*	*F*	*p*
	pF:ConVSTrt:PlasticType	1.9e-04	9.48e-05	2	24	0.00	1
	pF:ConVSTrt:ParticleSize	1.46e-01	7.31e-02	2	24	0.38	0.691
	Timepoint:pF:ConVSTrt	7.58e-02	7.58e-02	1	24	0.39	0.539
	pF:ConVSTrt:PlasticType:ParticleSize	3.5e-01	1.75e-01	2	24	0.90	0.421
	Timepoint:pF:ConVSTrt:PlasticType	5.26e-01	2.63e-01	2	24	1.35	0.278
	Timepoint:pF:ConVSTrt:ParticleSize	2.07e-02	1.04e-02	2	24	0.05	0.948
	Timepoint:pF:ConVSTrt:PlasticType:ParticleSize	1.3e-01	6.5e-02	2	24	0.33	0.72
Specific lipase activity (heptanoate)	Timepoint	4.91e+03	4.91e+03	1	24	24.94	< 0.001
	pF	3.08e+02	3.08e+02	1	24	1.57	0.223
	ConVSTrt	3.04e+02	3.04e+02	1	24	1.54	0.226
	pF:ConVSTrt	2.36e+01	2.36e+01	1	24	0.12	0.732
	Timepoint:pF	2.75e+01	2.75e+01	1	24	0.14	0.712
	Timepoint:ConVSTrt	6.12e+02	6.12e+02	1	24	3.11	0.091
	pF:ConVSTrt:PlasticType	4.16e+01	2.08e+01	2	24	0.11	0.9
	pF:ConVSTrt:ParticleSize	1.52e+03	7.62e+02	2	24	3.87	0.035
	Timepoint:pF:ConVSTrt	6.24e+02	6.24e+02	1	24	3.17	0.088
	pF:ConVSTrt:PlasticType:ParticleSize	2.04e+02	1.02e+02	2	24	0.52	0.603
	Timepoint:pF:ConVSTrt:PlasticType	3.63e+02	1.82e+02	2	24	0.92	0.411
	Timepoint:pF:ConVSTrt:ParticleSize	2.97e+01	1.49e+01	2	24	0.08	0.928
	Timepoint:pF:ConVSTrt:PlasticType:ParticleSize	3.05e+02	1.52e+02	2	24	0.77	0.472
Specific lipase activity (oleate)	Timepoint	9.15e-01	9.15e-01	1	24	1.65	0.212
	pF	6.46e+00	6.46e+00	1	24	11.63	0.002
	ConVSTrt	3.52e-01	3.52e-01	1	24	0.63	0.434
	pF:ConVSTrt	1.47e-01	1.47e-01	1	24	0.26	0.612
	Timepoint:pF	1.56e-03	1.56e-03	1	24	0.00	0.958
	Timepoint:ConVSTrt	1.58e+00	1.58e+00	1	24	2.84	0.105
	pF:ConVSTrt:PlasticType	1.24e+00	6.21e-01	2	24	1.12	0.343
	pF:ConVSTrt:ParticleSize	7.02e-01	3.51e-01	2	24	0.63	0.54
	Timepoint:pF:ConVSTrt	1.37e-02	1.37e-02	1	24	0.02	0.876
	pF:ConVSTrt:PlasticType:ParticleSize	4.57e-01	2.28e-01	2	24	0.41	0.668

Variable	Term	sumsq	meansq	*df num*	*df den*	*F*	*p*
	Timepoint:pF:ConVSTrt:PlasticType	2.76e-02	1.38e-02	2	24	0.02	0.976
	Timepoint:pF:ConVSTrt:ParticleSize	1.24e-01	6.22e-02	2	24	0.11	0.895
	Timepoint:pF:ConVSTrt:PlasticType:ParticleSize	6.54e-01	3.27e-01	2	24	0.59	0.563
Sum of microbial PLFAs	Timepoint	3.1e+02	3.1e+02	1	24	122.51	< 0.001
	pF	1.14e+02	1.14e+02	1	24	45.12	< 0.001
	ConVSTrt	1.94e+00	1.94e+00	1	24	0.77	0.39
	pF:ConVSTrt	4.42e-01	4.42e-01	1	24	0.17	0.68
	Timepoint:pF	1.05e+01	1.05e+01	1	24	4.14	0.053
	Timepoint:ConVSTrt	9.04e-02	9.04e-02	1	24	0.04	0.852
	pF:ConVSTrt:PlasticType	3.92e+00	1.96e+00	2	24	0.77	0.472
	pF:ConVSTrt:ParticleSize	6.38e+00	3.19e+00	2	24	1.26	0.301
	Timepoint:pF:ConVSTrt	3.84e-01	3.84e-01	1	24	0.15	0.7
	pF:ConVSTrt:PlasticType:ParticleSize	4.27e+00	2.14e+00	2	24	0.84	0.442
	Timepoint:pF:ConVSTrt:PlasticType	4.64e-02	2.32e-02	2	24	0.01	0.991
	Timepoint:pF:ConVSTrt:ParticleSize	1.81e+00	9.07e-01	2	24	0.36	0.702
	Timepoint:pF:ConVSTrt:PlasticType:ParticleSize	7.72e+00	3.86e+00	2	24	1.53	0.238
Specific phenoloxidase activity	Timepoint	3.27e+04	3.27e+04	1	24	4.56	0.043
	pF	3.13e+04	3.13e+04	1	24	4.35	0.048
	ConVSTrt	3.23e+03	3.23e+03	1	24	0.45	0.509
	pF:ConVSTrt	7.18e+03	7.18e+03	1	24	1.00	0.327
	Timepoint:pF	7.28e+01	7.28e+01	1	24	0.01	0.921
	Timepoint:ConVSTrt	1.56e+04	1.56e+04	1	24	2.17	0.154
	pF:ConVSTrt:PlasticType	7.29e+03	3.64e+03	2	24	0.51	0.608
	pF:ConVSTrt:ParticleSize	6.53e+03	3.26e+03	2	24	0.45	0.64
	Timepoint:pF:ConVSTrt	3.19e+02	3.19e+02	1	24	0.04	0.835
	pF:ConVSTrt:PlasticType:ParticleSize	2.7e+02	1.35e+02	2	24	0.02	0.981
	Timepoint:pF:ConVSTrt:PlasticType	1.77e+04	8.84e+03	2	24	1.23	0.31
	Timepoint:pF:ConVSTrt:ParticleSize	2.73e+04	1.36e+04	2	24	1.90	0.171
	Timepoint:pF:ConVSTrt:PlasticType:ParticleSize	6.48e+03	3.24e+03	2	24	0.45	0.642
	Timepoint	1.95e-01	1.95e-01	1	24	1.16	0.292

Variable	Term	sumsq	meansq	*df num*	*df den*	*F*	*p*
Specific ß-xylosidase activity	pF	1.51e-01	1.51e-01	1	24	0.90	0.353
	ConVSTrt	5.92e-01	5.92e-01	1	24	3.51	0.073
	pF:ConVSTrt	2.19e-02	2.19e-02	1	24	0.13	0.722
	Timepoint:pF	2.6e-01	2.6e-01	1	24	1.54	0.226
	Timepoint:ConVSTrt	1.11e-01	1.11e-01	1	24	0.66	0.425
	pF:ConVSTrt:PlasticType	3.28e-01	1.64e-01	2	24	0.97	0.393
	pF:ConVSTrt:ParticleSize	1.79e+00	8.94e-01	2	24	5.31	0.012
	Timepoint:pF:ConVSTrt	5.22e-03	5.22e-03	1	24	0.03	0.862
	pF:ConVSTrt:PlasticType:ParticleSize	1.71e-01	8.53e-02	2	24	0.51	0.609
	Timepoint:pF:ConVSTrt:PlasticType	1.86e-01	9.31e-02	2	24	0.55	0.583
	Timepoint:pF:ConVSTrt:ParticleSize	9.05e-02	4.52e-02	2	24	0.27	0.767
	Timepoint:pF:ConVSTrt:PlasticType:ParticleSize	2.35e-02	1.18e-02	2	24	0.07	0.933

Table S6-5. Comparisons of marginal means of control and PHB using the Tukey method (main effects according to significant *F* statistics). The difference between the marginal means of the control and PHB is expressed as absolute and relative deviation (%) from the estimated marginal mean of the control.

Variable	Contrast	Difference	Difference (%)	*df*	*t*	*p*
General bacterial PLFAs	PHB vs. Control	0.24 nmol g^{-1} dry soil	10.4	9.00	3.19	0.011
Gram-negative PLFAs	PHB vs. Control	0.35 nmol g^{-1} dry soil	18.6	9.00	6.44	< 0.001

Table S6-6. Comparisons of marginal means of control and PHB by soil moisture and timepoint levels using the Tukey method (interaction effects according to significant *F* statistics). The difference between the marginal means of the control and PHB is expressed as absolute and relative deviation (%) from the estimated marginal mean of the control.

Variable	Contrast	Factor level	Difference	Difference (%)	*df*	*t*	*p*
Gram-positive PLFAs	PHB vs. Control	Dry	2.35 nmol g^{-1} dry soil	25.9	9.00	3.21	0.011
		Wet	-0.09 nmol g^{-1} dry soil	-1.2	9.00	-0.17	0.872
Sum of microbial PLFAs		Dry	4.37 nmol g^{-1} dry soil	17.0	9.00	3.65	0.005
		Wet	0.73 nmol g^{-1} dry soil	3.2	9.00	0.79	0.449
Specific microbial respiration rate		104 days	0.01 µg CO_2 $nmol^{-1}$ PLFAs h^{-1}	30.5	18.00	3.57	0.002
		230 days	-0.00 µg CO_2 $nmol^{-1}$ PLFAs h^{-1}	-1.0	18.00	-0.12	0.909

Table S6-7. Comparisons of marginal means of timepoint and soil moisture using the Tukey method (main effects according to significant *F* statistics). The difference between the marginal means in wet and dry soil (pF = 2 / pF = 4) and at 104 and 230 days, respectively, is expressed as absolute and relative deviation (%) from the estimated marginal mean of dry soil and of 104 days, respectively.

Variable	**Contrast**	**Difference**	**Difference (%)**	***df***	***t***	***p***
Fungal PLFAs	230 vs. 104 days	-0.18 nmol g^{-1} dry soil	-23.8	24	-9.00	< 0.001
	Wet vs. dry	-0.17 nmol g^{-1} dry soil	-21.7	24	-7.71	< 0.001
Ratio of fungal to bacterial PLFAs	230 vs. 104 days	-0.01	-15.5	24	-4.30	< 0.001
	Wet vs. dry	-0.01	-11.5	24	-3.05	0.005
Ratio of Gram-positive to Gram-negative PLFAs	230 vs. 104 days	1.11	27.3	24	7.50	< 0.001
	Wet vs. dry	0.46	10.4	24	2.73	0.012
Gram-negative PLFAs	230 vs. 104 days	-0.55 nmol g^{-1} dry soil	-25.6	24	-14.66	< 0.001
	Wet vs. dry	-0.39 nmol g^{-1} dry soil	-18.8	24	-10.32	< 0.001
Specific lipase activity (oleate)	Wet vs. dry	0.55 nmol $nmol^{-1}$ PLFAs h^{-1}	16.9	24	2.21	0.037
Specific lipase activity (heptanoate)	230 vs. 104 days	-9.15 nmol $nmol^{-1}$ PLFAs h^{-1}	-11.4	24	-1.95	0.063
Specific phenoloxidase activity	230 vs. 104 days	71.4 nmol $nmol^{-1}$ PLFAs h^{-1}	23.6	24	2.52	0.019
	Wet vs. dry	67.99 nmol $nmol^{-1}$ PLFAs h^{-1}	22.3	24	2.25	0.034
Sum of microbial PLFAs	230 vs. 104 days	-4.19 nmol g^{-1} dry soil	-16.1	24	-7.87	< 0.001
	Wet vs. dry	-2.76 nmol g^{-1} dry soil	-10.9	24	-5.19	< 0.001

Table S6-8. Comparisons of marginal means of microbial variables in wet compared to dry soil by Timepoint (104 and 230 days) using the Tukey method. The difference between the marginal means of wet and dry soil (pF = 2 / pF = 4) is expressed as absolute and relative deviation (%) from the marginal mean of dry soil.

Variable	Contrast	Timepoint	Difference	Difference (%)	*df*	*t*	*p*
General bacterial PLFAs	Wet vs. dry	104 days	-0.18 nmol g^{-1} dry soil	-6.7	48.00	-2.36	0.022
		230 days	-0.38 nmol g^{-1} dry soil	-16.9	48.00	-5.17	< 0.001
Sum of bacterial PLFAs		104 days	-0.94 nmol g^{-1} dry soil	-6.8	48.00	-1.89	0.065
		230 days	-2.31 nmol g^{-1} dry soil	-17.7	48.00	-4.67	< 0.001
Specific ß-glucosidase activity		104 days	2.11 nmol $nmol^{-1}$ PLFAs h^{-1}	19.4	45.42	1.20	0.235
		230 days	-5.47 nmol $nmol^{-1}$ PLFAs h^{-1}	-28.8	45.42	-3.12	0.003
Specific microbial respiration rate		104 days	0.01 µg CO_2 $nmol^{-1}$ PLFAs h^{-1}	55.0	48.00	8.88	< 0.001
		230 days	0.01 µg CO_2 $nmol^{-1}$ PLFAs h^{-1}	78.1	48.00	11.49	< 0.001
Gram-positive PLFAs		104 days	-0.40 nmol g^{-1}dry soil	-4.5	48.00	-0.98	0.332
		230 days	-1.50 nmol g^{-1}dry soil	-16.9	48.00	-3.66	< 0.001

Table S6-9. Comparisons of marginal means of microbial variables at 230 days compared to 104 days by soil moisture level (dry / wet soil: pF = 4 / pF = 2) using the Tukey method. The difference between the marginal means at 104 and 230 days is expressed as absolute and relative deviation (%) from the marginal mean of 104 days.

Variable	Contrast	Soil moisture level	Difference	Difference (%)	*df*	*t*	*p*
General bacterial PLFAs	230 vs. 104 days	Dry	-0.33 nmol g^{-1} dry soil	-12.5	24.00	-3.87	< 0.001
		Wet	-0.54 nmol g^{-1} dry soil	-22.0	24.00	-8.56	< 0.001
Sum of bacterial PLFAs		Dry	-0.79 nmol g^{-1} dry soil	-5.7	24.00	-1.40	0.173
		Wet	-2.16 nmol g^{-1} dry soil	-16.8	24.00	-5.19	< 0.001
Specific ß-glucosidase activity		Dry	8.10 nmol $nmol^{-1}$ PLFAs h^{-1}	74.2	24.00	4.66	< 0.001
		Wet	0.52 nmol $nmol^{-1}$ PLFAs h^{-1}	4.0	24.00	0.40	0.693
Specific microbial respiration rate		Dry	-0.00 µg CO_2 $nmol^{-1}$ PLFAs h^{-1}	-8.9	24.00	-1.27	0.217
		Wet	0.00 µg CO_2 $nmol^{-1}$ PLFAs h^{-1}	4.7	24.00	1.38	0.180
Gram-positive PLFAs		Dry	0.06 nmol g^{-1} dry soil	0.7	24.00	0.12	0.902
		Wet	-1.04 nmol g^{-1} dry soil	-12.3	24.00	-3.01	0.006

Table S6-10. Comparisons of marginal means of lipase activities on the surface of MP particles by plastic type and soil moisture (dry / wet soil: pF = 4 / pF = 2), respectively. Statistical tests are performed on log-transformed data. The ratios of the contrasts are back-transformed to the original scale of the data.

Contrast	**Factor level**	**Ratio**	***df***	***t* ratio**	***p***
pF 4 / pF 2	PLA/PBAT	3.69	11	5.02	< 0.001
pF 4 / pF 2	LDPE	1.96	11	2.81	0.017
(PLA/PBAT) / LDPE	pF 4	7.45	11	7.60	< 0.001
(PLA/PBAT) / LDPE	pF 2	3.91	11	5.90	< 0.001

Supplementary figures

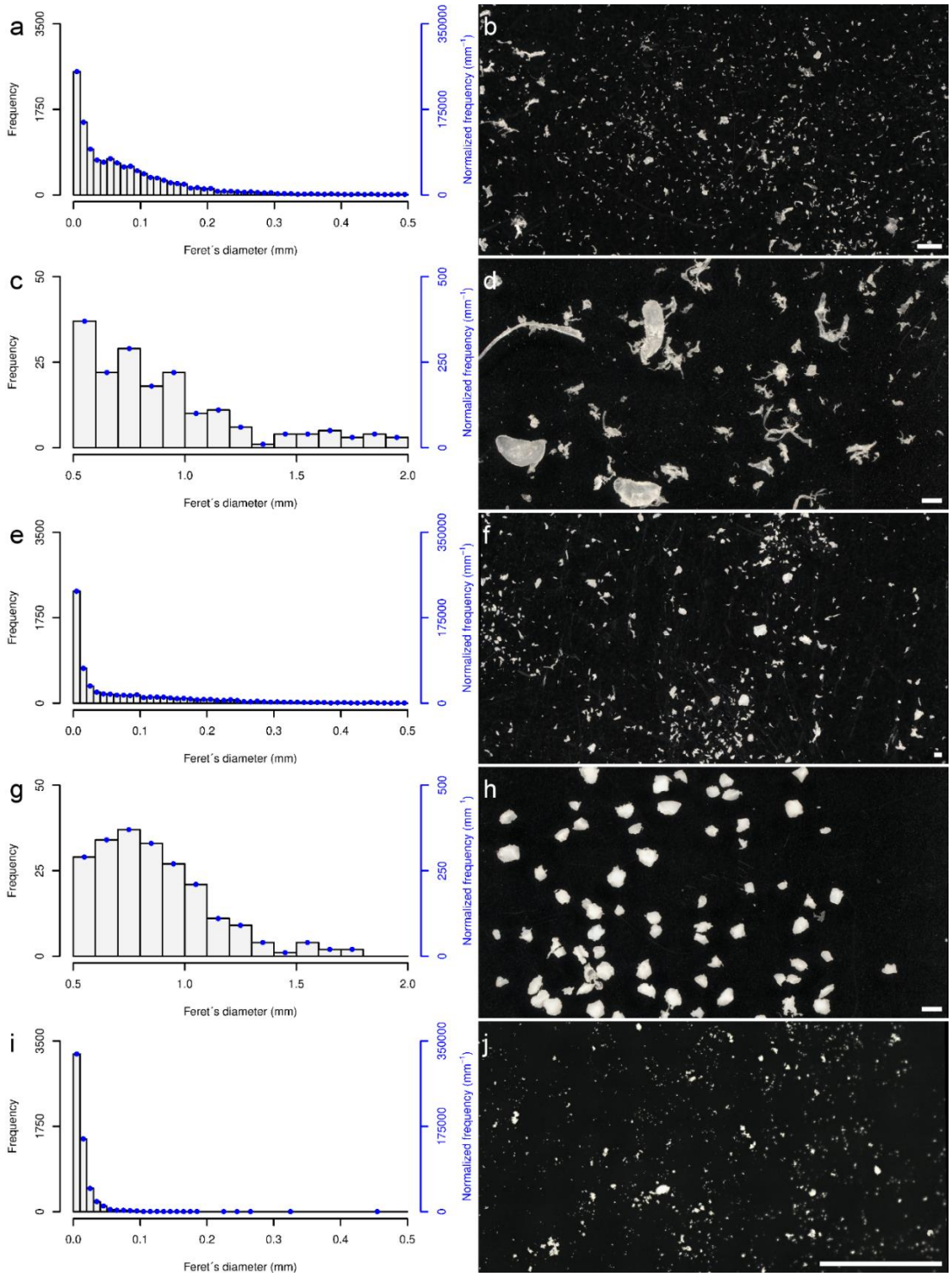


Fig. S6-1. Particle size distributions and representative micrographs of MP used in this study. **a** – **b** LDPE < 0.5 mm. **c** – **d** LDPE 0.5 – 2 mm. **e** – **f** PLA/PBAT < 0.5 mm. **g** – **h** PLA/PBAT 0.5 - 2 mm. **i** – **j** PHB. Note that particles < 3 µm were excluded from the particle size distributions due to the lower detection limit of a light microscope (Shekunov et al. 2007). Particles outside the mesh size range of the sieves were also not considered in the particle size distribution. Normalized frequencies were calculated according to Filella (2015). Scale bars: 1 mm.

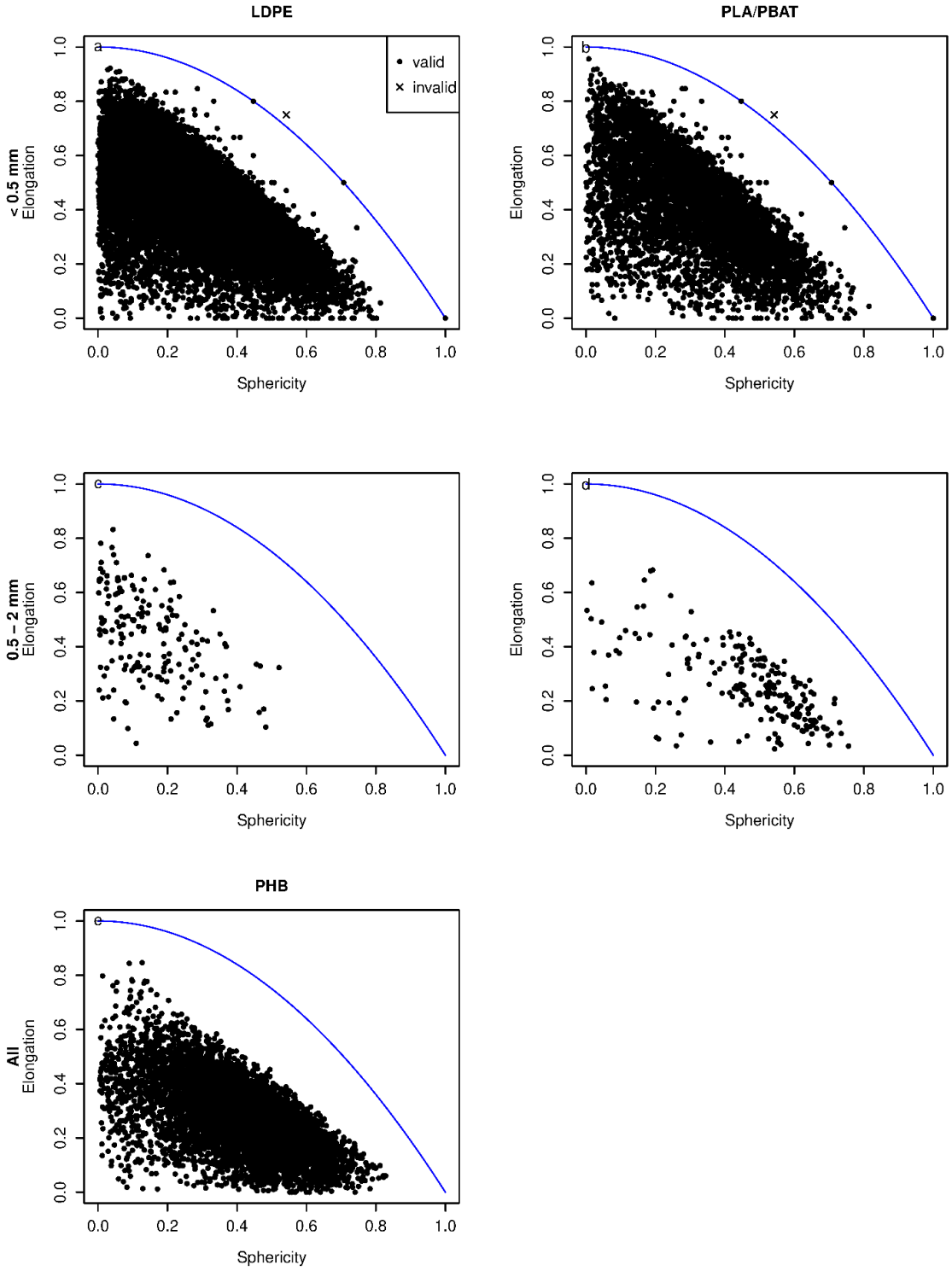


Fig. S6-2. Sphericity versus Elongation diagram for MP used in this study (Kröner and Doménech Carbó 2013). **a** LDPE < 0.5 mm. **b** PLA/PBAT < 0.5 mm. **c** LDPE 0.5 – 2 mm. **d** PLA/PBAT 0.5 – 2 mm. **e** PHB. The blue line indicates the theoretical maximum of elongation for a given sphericity. Most MP particles had a valid combination of sphericity and elongation (points). Only very few particles are pictured as invalid on the right of the theoretical curve due to digitization errors (crosses). This indicates that the digital resolution of the images was sufficient for shape characterization.

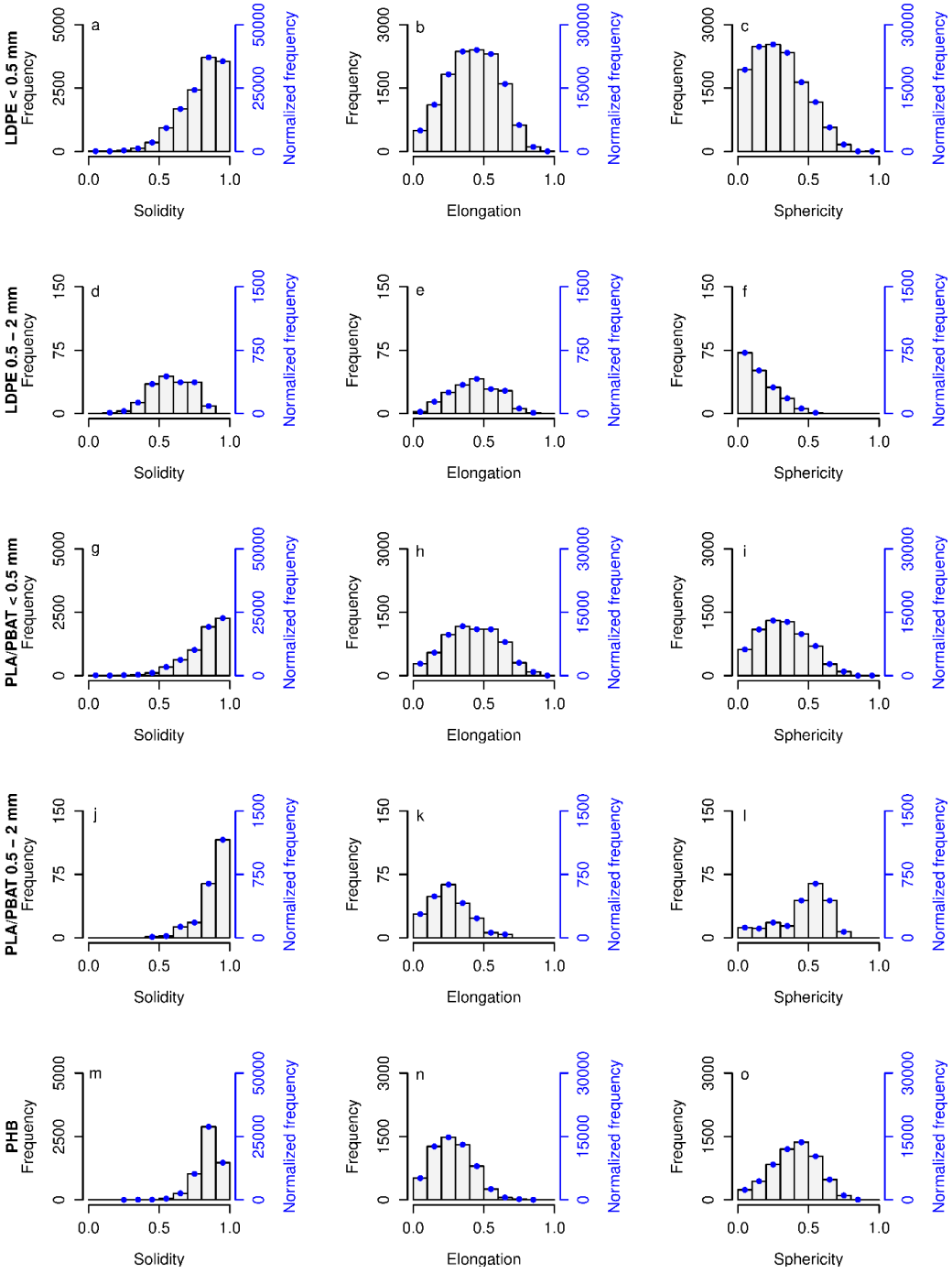


Fig. S6-3. Shape distributions for sphericity, elongation, and solidity of MP used in this study. **a** – **c** LDPE < 0.5 mm. **d** – **f** LDPE 0.5 – 2 mm. **g** – **i** PLA/PBAT 0.5 mm. **j** – **l** PLA/PBAT 0.5 – 2 mm. **m** – **o** PHB. Sphericity is the parameter that indicates how much a MP particle deviates from a perfect circle. Thus, values closer to unity indicate a spherical shape. The aspect ratio of MP was measured in terms of elongation. Values closer to unity indicate a very small breadth-to-length ratio, while values closer to zero are typical for particles with a circular shape. Note that particles with either a rough or a smooth surface might have equal elongations. To determine surface ruggedness of particles, solidity was assessed. Values close to unity are typical for smooth surfaces, while values closer to zero tend to be found in case of irregularly shaped particles. All shape descriptors were calculated by formulae mentioned in the literature (Crompton 2005; Riley 1941). Note that values for shape descriptors were removed when they fell outside the theoretical range of the Sphericity versus Elongation model (Kröner and Doménech Carbó 2013; Fig. S6-2). Normalized frequencies were calculated according to Filella (2015).

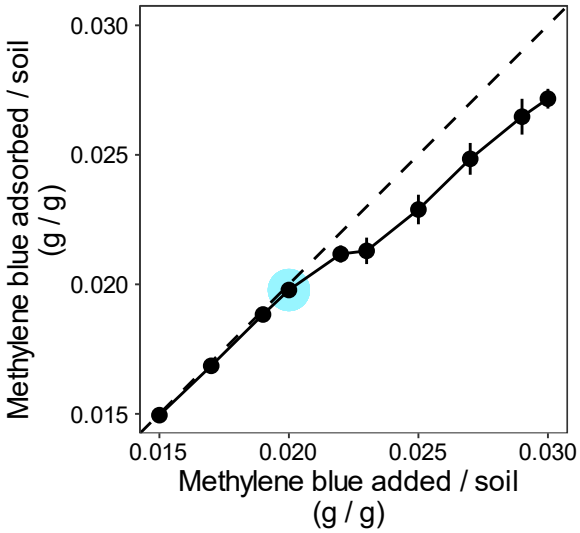


Fig. S6-4. Determination of the specific soil surface area via the methylene blue method according to Yukselen and Kaya (2006). Methylene blue absorbed by soil against methylene blue added to the soil. Points represent the mean value of three replicates (except at 0.015 g Methylene blue added to soil, with *n* = 2, where one replicate was below the limit of quantification). The bright blue stroke indicates the point of full cation replacement. Error bars show standard errors.

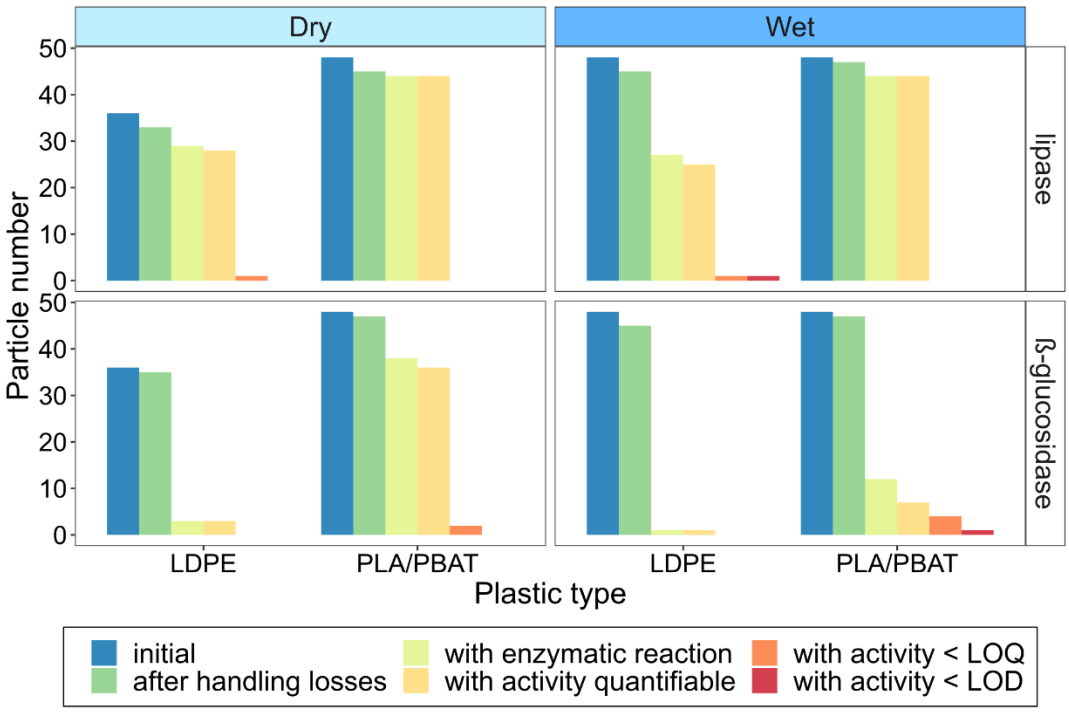


Fig. S6-5-. Number of MP particles used in the lipase and ß-glucosidase analyses on the surface of MP particles. Number of MP particles: initially used in the enzyme assay, after handling losses, showing an enzymatic reaction, with quantifiable enzyme activity, with enzyme activity below the limit of quantification (LOQ), and below the limit of detection (LOD), respectively, in dry (pF = 4) and wet soil (pF = 2).

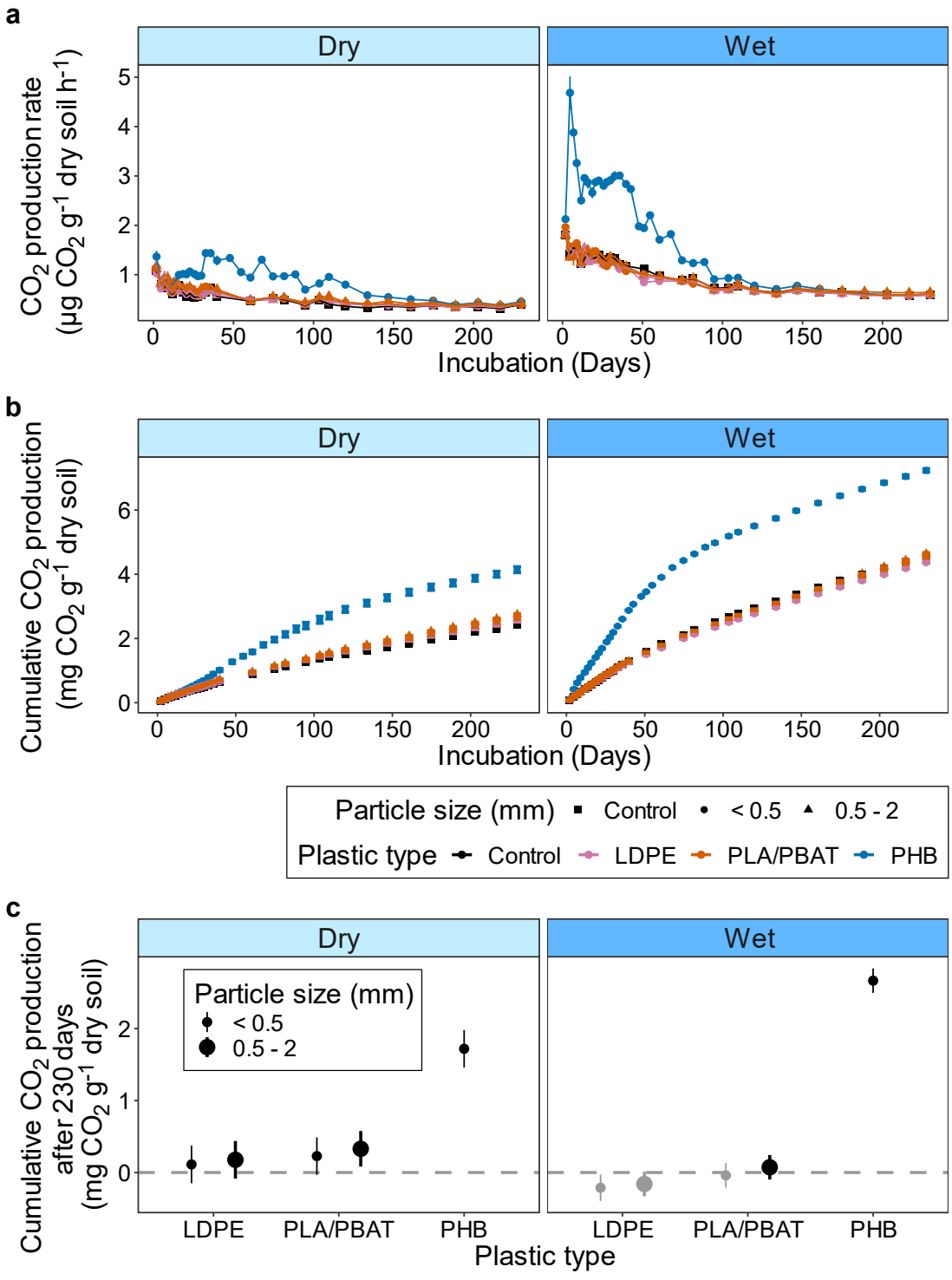
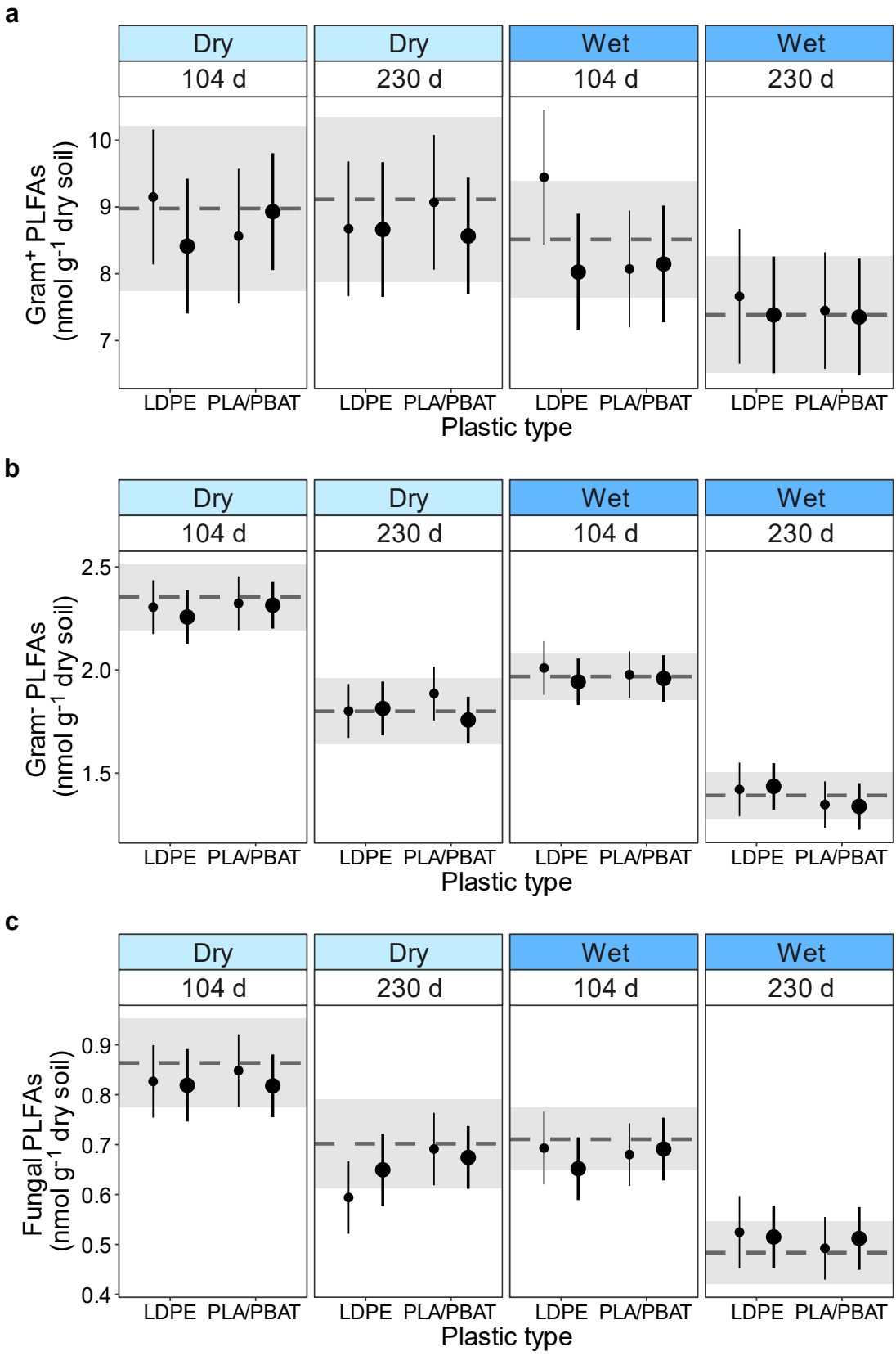
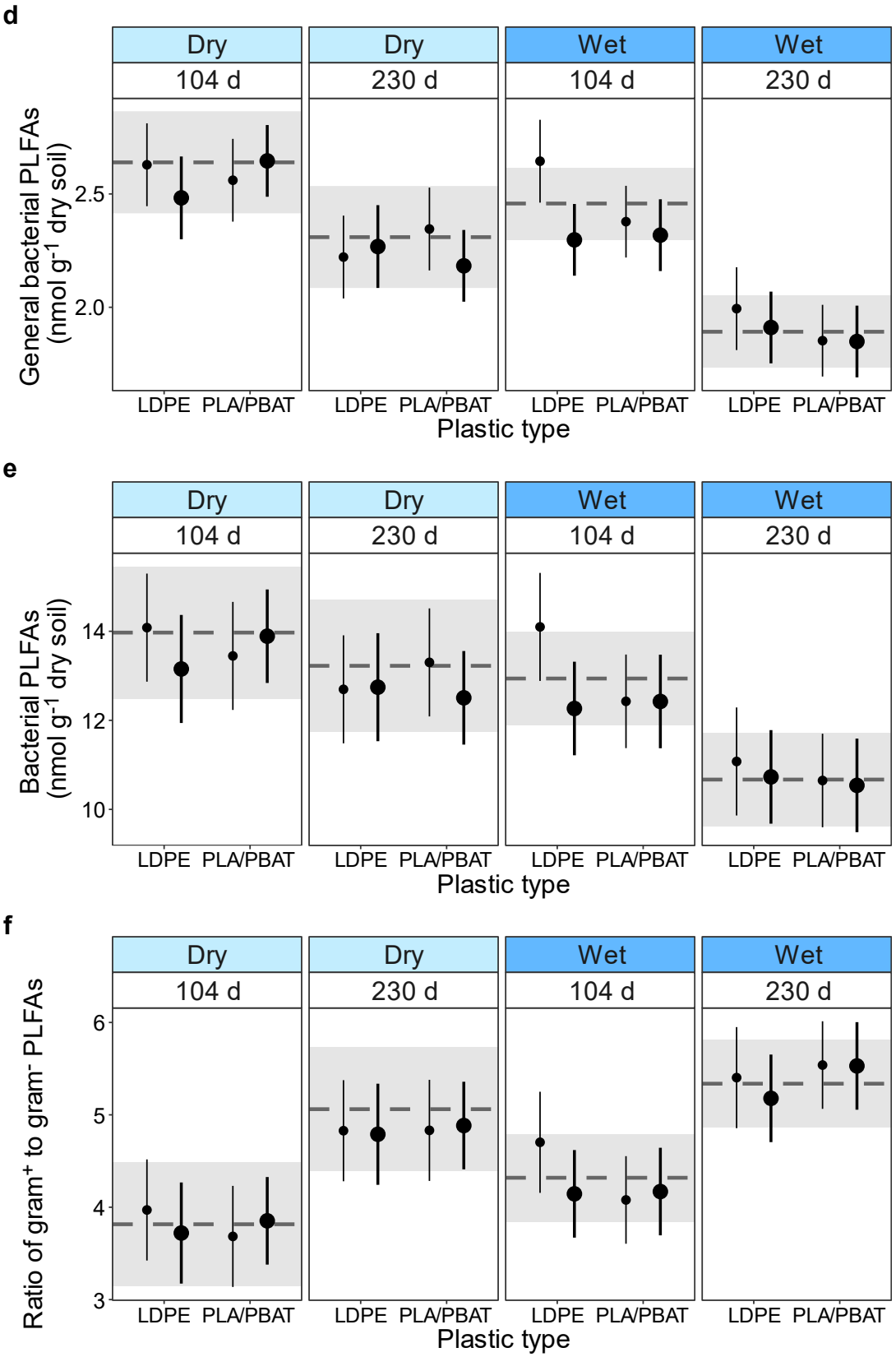
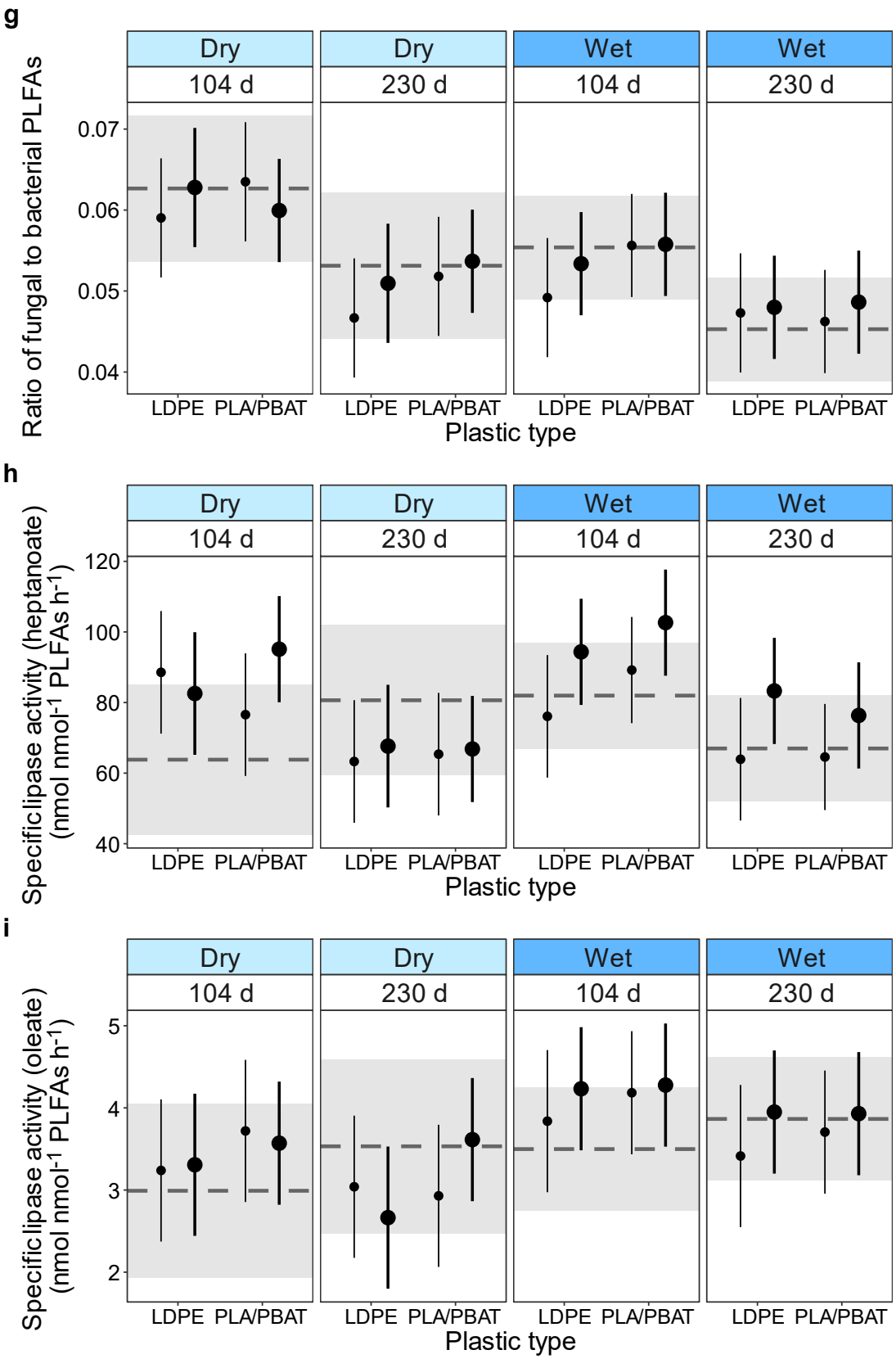


Fig. S6-6. a CO_2 production rates and **b** cumulative CO_2 production over 230 days in response to small and large (< 0.5 / 0.5 - 2 mm) LDPE, PLA/PBAT, and PHB (< 0.5 mm, positive control) particles in dry and wet soil (pF = 4/ pF = 2). Error bars show standard errors. **c** Differences in cumulative CO_2 production after 230 days between MP-amended soil compared to MP-free control soil. Error bars show 95% CIs obtained from two-tailed Dunnett's tests. Gray-colored figures indicate that cumulative CO_2 production was lower than that of the corresponding MP-free control soil.


a
Dry
104 d
Dry
230 d
Wet
104 d
Wet
230 d
Gram+ PLFAs (nmol g-1 dry soil)
10
9
8
7
LDPE PLA/PBAT
Plastic type
b
Gram- PLFAs (nmol g-1 dry soil)
2.5
2.0
1.5
c
Fungal PLFAs (nmol g-1 dry soil)
0.9
0.8
0.7
0.6
0.5
0.4


d
Dry
104 d
Dry
230 d
Wet
104 d
Wet
230 d
General bacterial PLFAs (nmol g-1 dry soil)
2.5
2.0
LDPE PLA/PBAT
Plastic type
e
Bacterial PLFAs (nmol g-1 dry soil)
14
12
10
f
Ratio of gram+ to gram- PLFAs
6
5
4
3


g
Dry
104 d
Dry
230 d
Wet
104 d
Wet
230 d
Ratio of fungal to bacterial PLFAs
0.07
0.06
0.05
0.04
LDPE PLA/PBAT
Plastic type
h
Specific lipase activity (heptanoate)
(nmol nmol-1 PLFAs h-1)
120
100
80
60
40
i
Specific lipase activity (oleate)
(nmol nmol-1 PLFAs h-1)
5
4
3
2

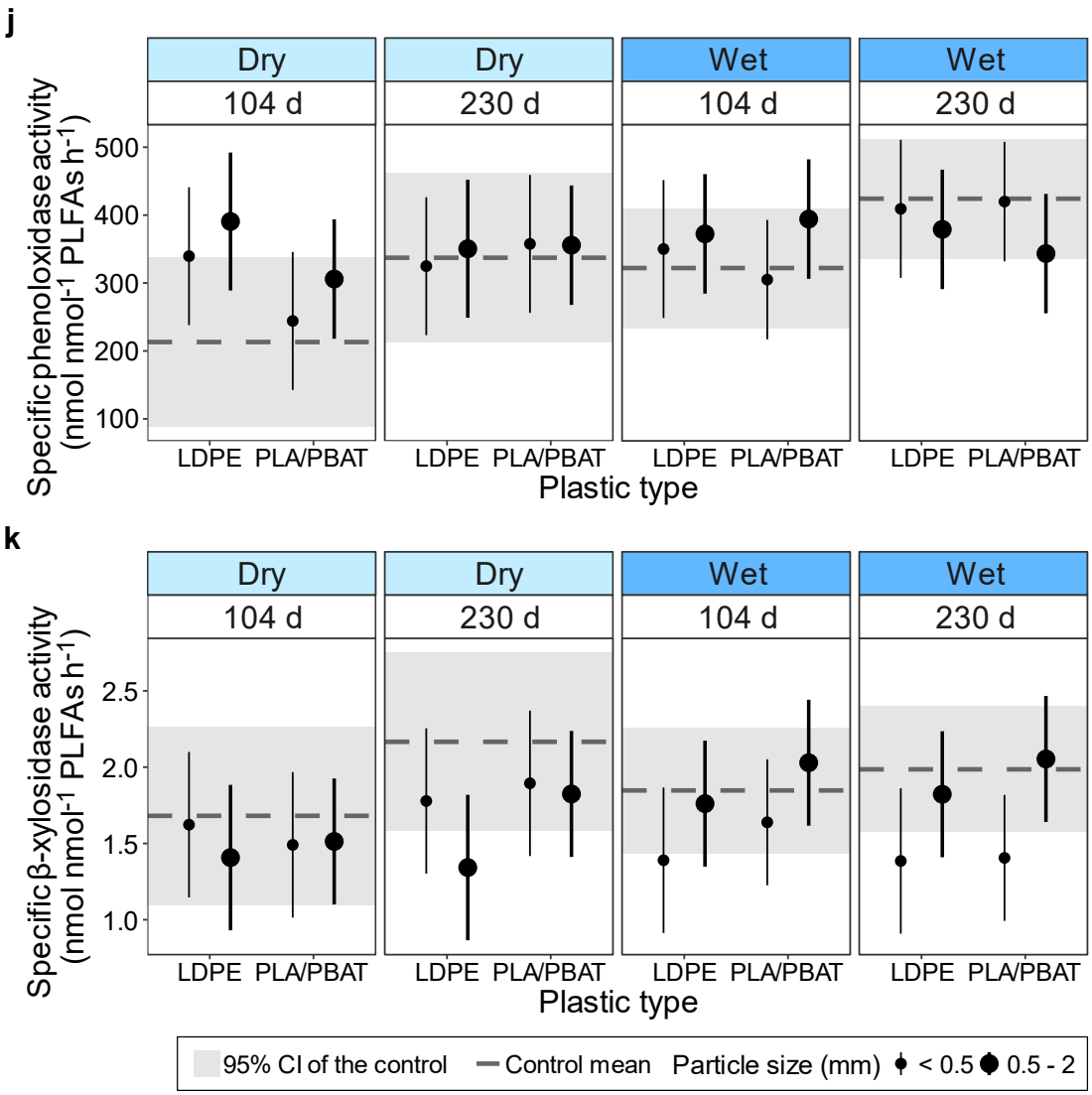


Fig. S6-7. Composition and abundance of main microbial groups and specific enzyme activity in response to small and large (< 0.5 / 0.5-2 mm) LDPE and PLA/PBAT particles in dry and wet soil (pF = 4/ pF = 2) after 104 and 230 days in comparison to MP-free soil (control). **a** Gram-positive, **b** Gram-negative, **c** fungal, **d** general bacterial, **e** sum of bacterial PLFAs. **f** Ratio of Gram-positive to Gram-negative bacteria and **g** ratio of fungi to bacteria. **h** Specific lipase activity with heptanoate and **i** oleate as the substrate, respectively. **j** Specific phenoloxidase and **k** specific ß-xylosidase activity. Specific enzyme activities are related to the sum of microbial PLFAs (nmol) as indicator for microbial biomass. Data are presented as estimated marginal means and 95% confidence intervals (error bars) obtained from linear mixed effect models. The dashed line and the gray-colored area display the mean and the 95% confidence intervals, respectively, of the MP-free control soil.

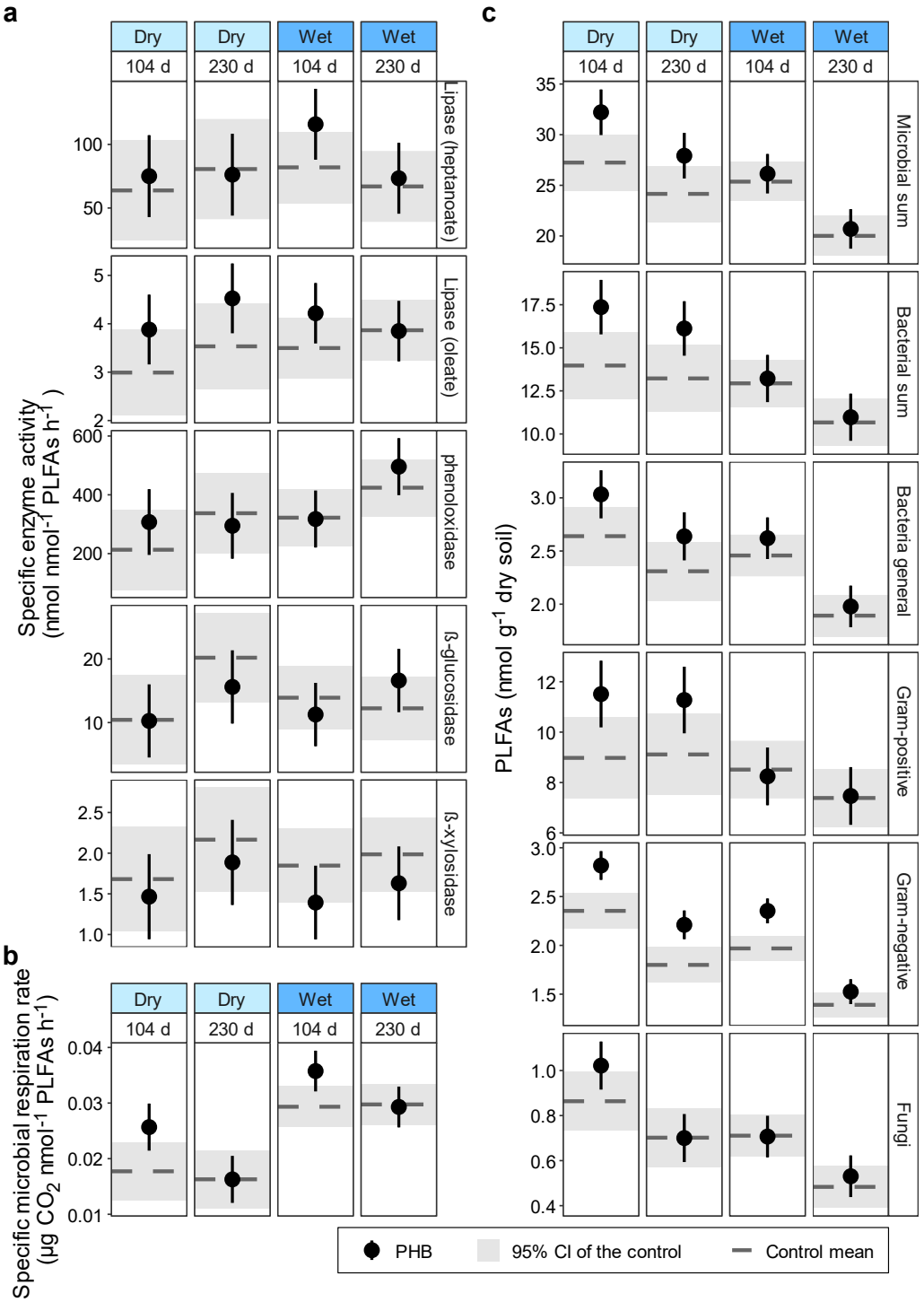


Fig. S6-8. **a** Specific enzyme activities, **b** specific microbial respiration rate, and **c** composition and abundance of main microbial groups in response to PHB addition to soil in relation to MP-free control soil. Specific microbial respiration rate and specific ß-glucosidase activity are both related to the sum of microbial PLFAs (nmol) as indicator for microbial biomass. Data are presented as estimated marginal means and 95% confidence intervals obtained from linear mixed effect models. The dashed line and the gray-colored area display the mean and the 95% confidence intervals, respectively, of the MP-free control soil.

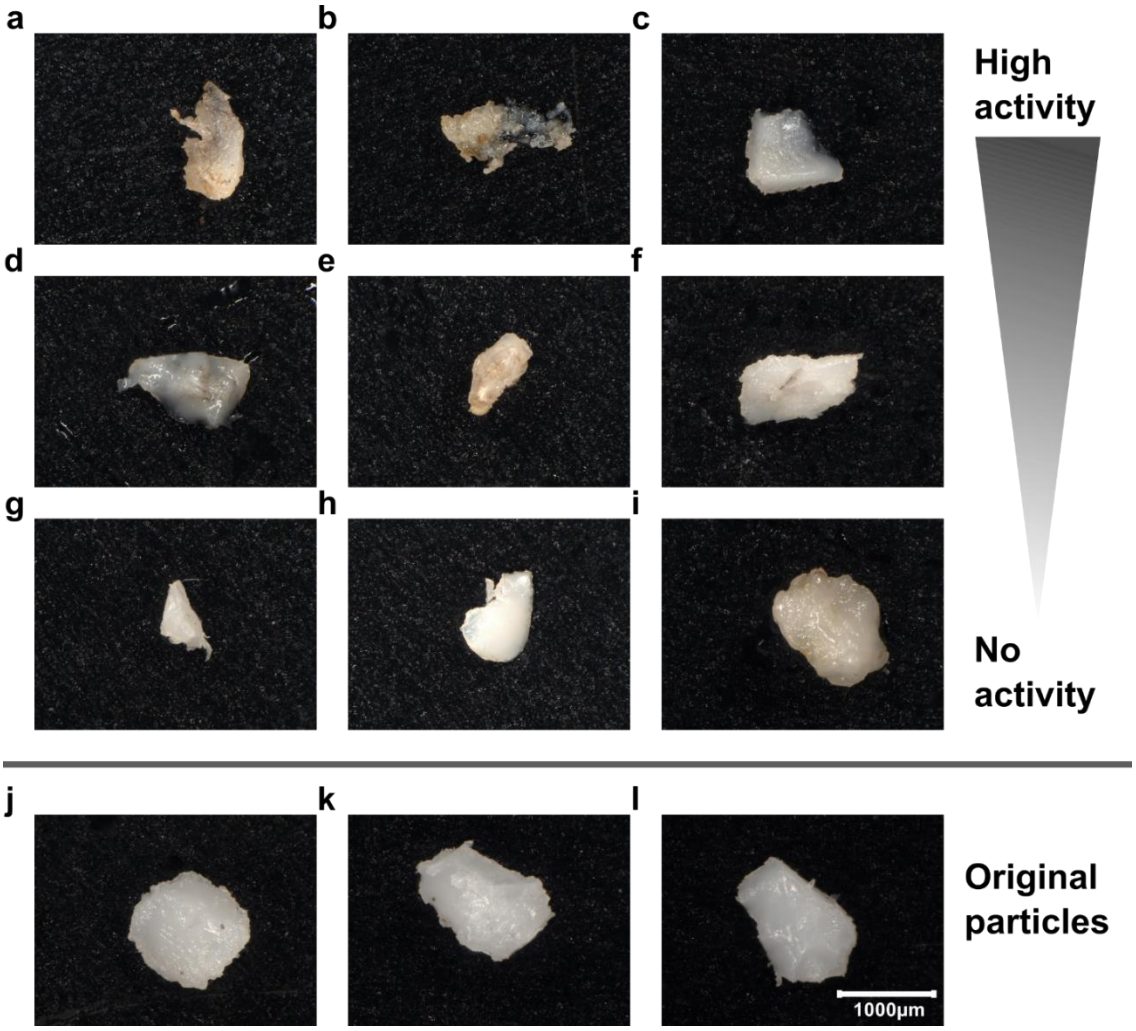
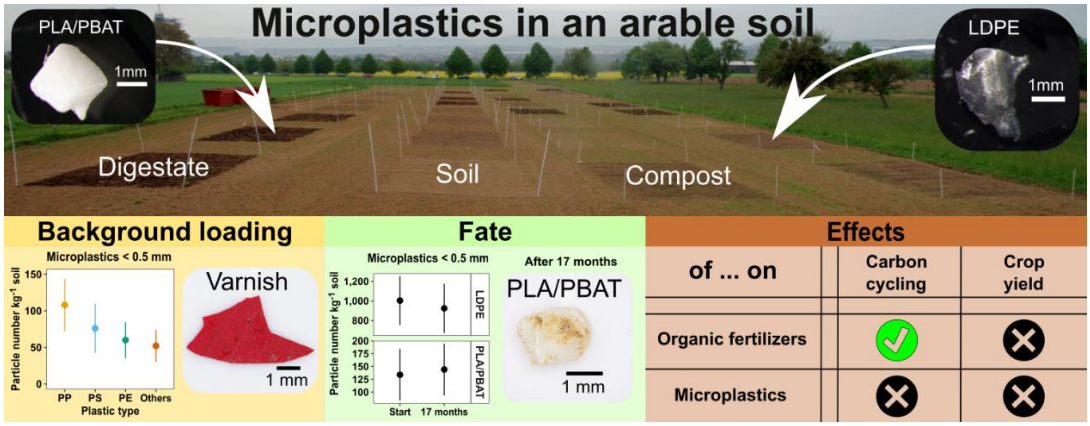


Fig. S6-9. PLA/PBAT particles after incubation for 230 days in dry soil (pF = 4). MP particles **a** – **c** with high lipase activity, **d** – **f** with lipase activity between 25th and 75th percentiles, and **g** – **i** with no quantifiable activity. **j** – **l** are non-incubated PLA/PBAT control particles.

7 Microplastics persist in an arable soil but do not affect soil microbial biomass, enzyme activities, and crop yield



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Keywords: field experiment, LDPE, microbial biomass, organic fertilizers, PLA/PBAT, plastics contamination, soil enzyme activity

7.1 Abstract

Background Microplastics (MP, plastic particles < 5 mm) are ubiquitous in arable soils due to significant inputs via organic fertilizers, sewage sludges, and plastic mulches. However, knowledge of typical MP loadings, their fate and ecological impacts on arable soils is limited.

Aims We studied (1) MP background concentrations, (2) the fate of added conventional and biodegradable MP, and (3) effects of MP in combination with organic fertilizers on microbial abundance and activity associated with carbon (C) cycling, and crop yields in an arable soil.

Methods On a conventionally managed soil (Luvisol, silt loam), we arranged plots in a randomized complete block design with the following MP treatments (none, low-density polyethylene [LDPE], a blend of poly(lactic acid) and poly(butylene adipate-co-terephthalate) [PLA/PBAT]), and organic fertilizers (none, compost, digestate). We added 20 kg MP ha^{-1} and 10 t organic fertilizers ha^{-1}. We measured concentrations of MP in the background and of added MP, microbiological indicators of C cycling (microbial biomass and enzyme activities), and crop yields over 1.5 years.

Results Background concentration of MP in the top 10 cm was 296 ± 110 (mean ± standard error) particles < 0.5 mm per kg soil, with polypropylene, polystyrene, and polyethylene as the main polymers. Added LDPE and PLA/PBAT particles showed no changes in number and particle size over time. MP did not affect the soil microbiological indicators of C cycling or crop yields.

Conclusions Numerous MP occur in arable soils, suggesting diffuse MP entry into soils. In addition to conventional MP, biodegradable MP may persist under field conditions. However, MP at current concentrations are not expected to affect C turnover and crop yield.

7.2 Introduction

Microplastics (MP) are commonly defined as plastic particles of various shapes and sizes between 100 nm and 5 mm (*Okoffo* et al., 2021; *de Souza Machado* et al., 2018). MP are suspected threats to soil organisms and functions (*Helmberger* et al., 2020; *Pathan* et al., 2020; *Rillig* et al., 2021; *Q. Wang* et al., 2022). Arable soils receive MP primarily due to amendment with sewage sludge, organic fertilizers, and plastic mulch (*Corradini* et al., 2019; *Gui* et al., 2021; *van Schothorst* et al., 2021; *Vithanage* et al., 2021; *J. Wang* et al., 2021; *Weithmann* et al., 2018; *Yang* et al., 2021). In addition, MP can enter soils through both wet and dry atmospheric deposition (*Allen* et al., 2019; *Brahney* et al., 2020; *Kernchen* et al., 2022). Soils receiving MP via sewage sludge application and plastic mulching have a global median background concentration of 1,200 particles kg^{-1} soil (*Büks* and *Kaupenjohann*, 2020).

Similarly, *van Schothorst* et al. (2021) found on average 888 particles kg^{-1} in soils that received annual compost inputs of 10 t ha^{-1} in the past 7 – 20 years. However, the reported uncertainties are large; robust estimates of MP loadings in soils due to organic fertilizer application are therefore not available (*Büks* and *Kaupenjohann*, 2020; *Gui* et al., 2021).

Biowaste as well as the composts and digestates derived thereof have been found to contain plastics and there is some evidence plastic pieces can break down and form MP during biowaste processing (*Judy* et al., 2019; *Gui* et al., 2021; *Rodrigues* et al., 2020; *Watteau* et al., 2018; *Weithmann* et al., 2018). Composts contain plastics mainly from packaging and plastic bag residues, which are usually made up of low-density polyethylene (LDPE) (*Bandini* et al., 2020; *Gui* et al., 2021; *Rodrigues* et al., 2020; *Weithmann* et al., 2018). There have been attempts to tackle plastic contamination of composts and soils by replacing conventional plastics such as LDPE with biodegradable polymers (*Agarwal*, 2020; *Liao* and *Chen*, 2021; *Qin* et al., 2021). Polymer blends with poly(lactic acid) (PLA) and poly(butylene adipate-co-terephthalate) (PBAT) are biodegradable alternatives to LDPE (*Agarwal*, 2020; *Liao* and *Chen*, 2021; *Musioł* et al., 2018). LDPE is resistant to microbial degradation due to its stable carbon (C) backbone (*Kumar Sen* and *Raut*, 2015; *Krueger* et al., 2015). In contrast, PLA/PBAT blends are hydrolyzable through enzymes such as lipases, cutinases, and esterases, and thus potentially biodegradable in soil or compost (*Freitas* et al., 2017; *Jia* et al., 2021; *Palsikowski* et al., 2018; *Tabasi* and *Ajji*, 2015; *Weng* et al., 2013; *Zumstein* et al., 2018). However, there is significant uncertainty about the fate of biodegradable MP fragments originating from composts in arable soils. Indeed, there is some evidence for incomplete biodegradation of some biodegradable plastics, rapid fragmentation of biodegradable MP and thus more rapid in situ formation of MP in soil compared to conventional polymers (*Liao* and *Chen*, 2021; *Meng* et al., 2022; *Qin* et al., 2021; *Steiner* et al., 2022). Biodegradable polymers could thus pose a greater risk of adverse effects on soil organisms and functions if they are not readily mineralized.

MP have many modes of action in soils. They can induce physicochemical changes in habitats by affecting soil porosity, bulk density, water holding capacity, and soil water repellence (*X. Zhang* et al., 2021), and form specific habitats for soil microorganisms, referred to as the plastisphere (*Bandopadhyay* et al., 2020; *Rüthi* et al., 2020; *M. Zhang* et al., 2019; *Zhou* et al., 2021). Less is known about the influence of MP on C cycling, but MP are C-rich substrates and have the potential to change soil organic C and thus C cycling (*Meng* et al., 2022; *Rillig* et al., 2021; *X. Zhang* et al., 2021). Soil C cycling involves the decomposition of organic compounds originating from plant, microbial, and animal residues. The degradation of different complex compounds (cellulose, chitin < xylan < lignin) is catalyzed by microbially produced enzymes

(*Burns* et al., 2013). For example, ß-glucosidase and N-acetyl-glucosaminidase catalyze the final hydrolytic cleavage of cellobiose and chitobiose di- and oligomers after depolymerisation of cellulose and chitin (*Kandeler*, 2015; *Maillard* et al., 2018), whereas ß-xylosidase hydrolyzes cleavage products, e.g., xylobioses and other short xylooligosaccharides, from different hemicelluloses such as xylan (*Dodd* et al., 2011; *Uffen*, 1997). Phenoloxidases oxidize redox mediators initiating the depolymerisation of lignin (*Burns* et al., 2013).

The first study under field conditions observed increases in C cycling enzymes (α- and ß-glucosidase) in response to LDPE-MP addition (*Lin* et al., 2020). A recent meta-analysis identified multiple negative impacts on plant growth including crop yield and plant height, resulting from pollution of croplands with plastic residues from mulch films (*D. Zhang* et al., 2020). Given the importance of agricultural soils for food production, understanding the loadings and the extent to which MP, and especially biodegradable MP, affect C cycling and crop yields in agroecosystems is crucial (*Rillig* et al., 2017; *G. S. Zhang* and *Liu*, 2018; *X. Zhang* et al., 2021).

This study aimed to better understand the fate of MP and effects of MP on microbial abundance and activity related to C cycling, as well as crop yields in arable soils. We established a field experiment (1) to investigate MP background concentrations, (2) to quantify concentrations of added conventional and biodegradable MP after one and 17 months of addition, and (3) to identify potential effects of MP and of MP-containing organic fertilizers on soil microbial abundance, activities of selected C cycling enzymes, and crop yields. We expected that (1) the arable soil shows a low but significant background MP loading (before setup), (2) biodegradable MP (PLA/PBAT) fragment in soil, (3) conventional MP (LDPE) persist and are not altered, (4) biodegradation of PLA/PBAT leads to increased activity of lipase in soil as this enzyme catalyzes ester bond cleavage, but microbial abundance, activities of enzymes catalyzing other reactions, and crop yields are not affected because breakdown of PLA/PBAT is slow and direct toxic effects MP on plants are unlikely, and (5) due to its persistence, LDPE has no impact on soil microbiological indicators of C cycling or crop yields.

7.3 Material and methods

Microplastics

As biodegradable plastics we used a blend of poly(lactic acid) (PLA; IngeoTM Biopolymer 7001D, NatureWorks LLC, Minnetonka, MN, United States) and poly(butylene adipate-co-terephthalate) (PBAT; Ecoflex F Blend C1200, BASF SE, Ludwigshafen, Germany) in a mixing ratio of 80/20 % w/w, which was compounded at the Institut für Kunststofftechnik

(University of Stuttgart, Stuttgart, Germany). Low-density polyethylene (LDPE; Lupolen 2420H, LyondellBasell Industries N.V., Rotterdam, Netherlands) served as the representative conventional MP. Polymer pellets were cryomilled (-196 °C) with a speed rotor mill (Pulverisette, Fritsch GmbH, Idar-Oberstein, Germany) to obtain MP and subsequently fractionated using stainless-steel sieves to obtain two MP particle size fractions of < 0.5 mm and 0.5 – 2 mm. Both fractions were then mixed in a 1:1 ratio (mass-based).

Organic fertilizers

Solid digestate (C/N: 11, dry mass: 22.2 %, substrate: 48.8 % plant residues such as silage, 51.2 % animal by-products such as manure) was provided by the research station Unterer Lindenhof of the University of Hohenheim. Compost (C/N:17, dry mass: 61.8%, substrate: green cuttings) originated from Häckselplatz Möhringen in Stuttgart, Germany.

Since there were no detection methods for MP particles < 1 mm in composts and digestates at the initiation of the experiment (*Weithmann* et al., 2018), we used the plastic loading of the fractions 1 – 5 mm and > 5 mm in the compost and digestate as indicators of MP loading. The plastic loading of digestates and composts was determined after sieving and detection via attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy (c.f. section "Microplastics analyses"). The compost (one batch) contained three polypropylene (PP) particles in the fractions 1 – 5 mm per kg and three particles (50 % were PP and 50 % polystyrene [PS]) in the fraction > 5 mm per kg. The digestate (mean of two batches) contained 11 particles in the fraction > 5 mm per kg (25 % were PE and 75 % PP) and no particles in the 1 – 5 mm fraction.

Study site characteristics, experimental setup, and soil sampling

The experiment was established on a conventionally managed agricultural field at the research station Heidfeldhof (University of Hohenheim, central point of the field: 9°11'22.984" longitude, 48°43'11.137" latitude, EPSG: 4326, WGS 1984). In the past, neither plastic mulch nor compost had been applied. In addition to the mineral fertilizers commonly used in conventional management, the field was sporadically fertilized with manure from the research station Meiereihof (University of Hohenheim). The soil is a Luvisol with texture silt loam (3.4 % sand, 76.2 % silt, 20.5 % clay), total soil C and nitrogen (N) content of 1.19 and 0.13 %, respectively (C/N ratio: 9), and pH of 6.3 (measured in 0.01 M $CaCl_2$). Weather conditions at the study site and farm management during the experiment are shown in Fig. 7-1.

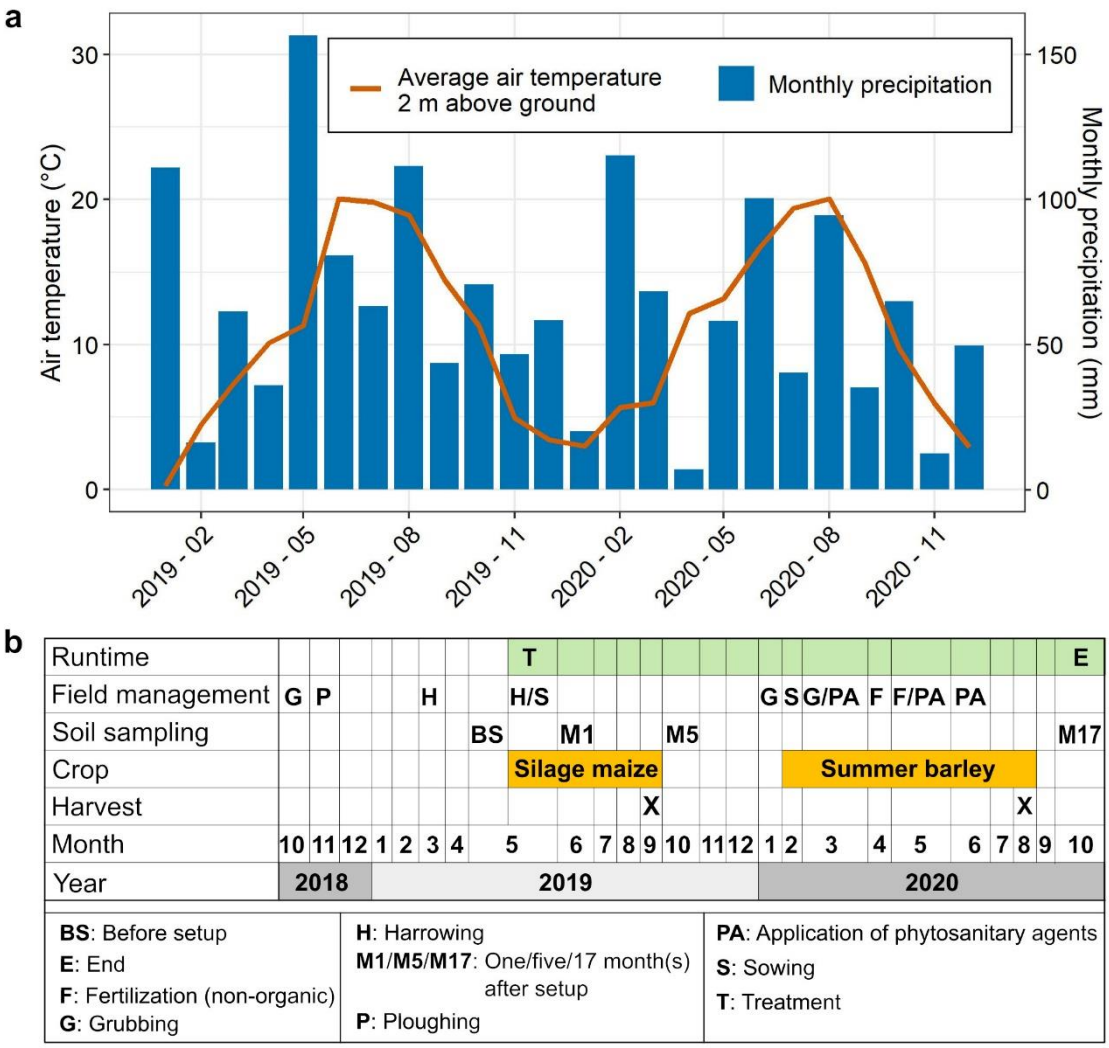

Fig. 7-1. a Monthly average air temperature measured in 2 m above ground and monthly precipitation from nearby meteorological station, **b** overview of field management, soil sampling, harvest. Meteorological data were obtained from *LTZ* (2021).

The experimental design included the factors MP (none, LDPE, PLA/PBAT) and organic fertilizer (none, compost, digestate) arranged in a complete randomized block design with four blocks (Fig. 7-2). The area of one plot was 32 m² (length: 8 m, width: 4 m). To avoid carryover effects from one plot to another by tillage, a 5 m – wide buffer area between the plots was established in the direction of machine travel. In consideration of German biowaste regulations that permits an application of max. 30 t compost ha^{-1} (note that all mass data are given on dry matter basis) over three years (*BioAbfV*, 2017), we applied 10 t ha^{-1} of compost and digestate. MP were applied at a concentration of 2 g m^{-2}. To homogeneously apply the MP, we weighted 10 kg soil randomly taken from the field per plot and added 68 g MP, then homogenized these MP-soil-mixtures using a drilling machine with a stirring unit for 2 min in metal buckets (35 L). From these MP-soil-mixtures, we took the amount required for two square meters, i.e., 0.59 kg,

added these to the plots (treatments without fertilizer) or mixed these with the amount of compost for two square meters, i.e., 2 kg, using a drilling machine (treatments with compost). We chose these MP-soil-mixtures and compost because they could be mixed, transported, and distributed well in the field. Due to the low bulk density of the digestate, it could not be mixed in the metal buckets with the MP-soil-mixtures. Therefore, we applied the digestate and MP-soil-mixtures (treatments with digestate and MP) separately to the field.

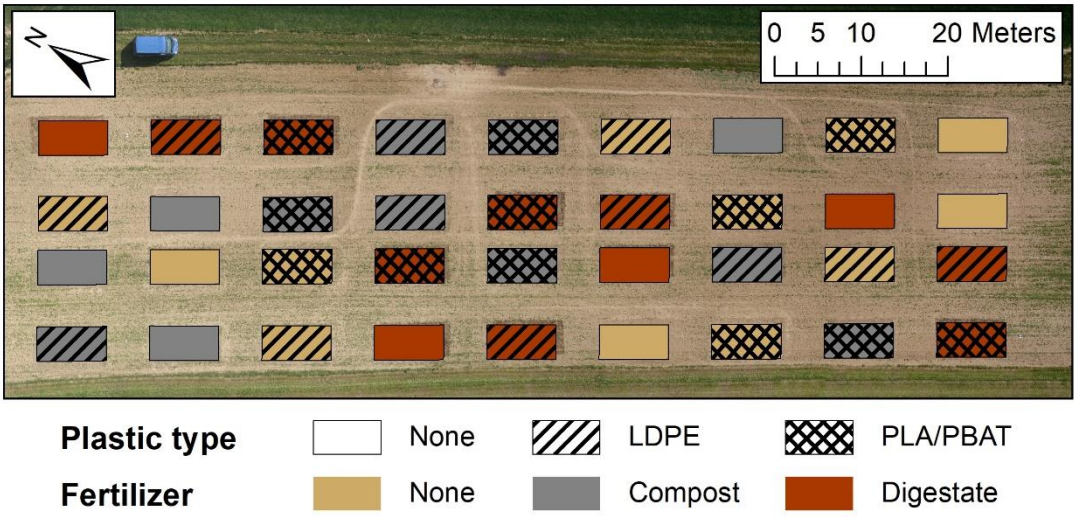

Fig. 7-2. Experimental design of the field experiment. Plots were arranged in a complete randomized block design (*n* = 4).

To investigate MP background contamination and determine soil properties, we took 15 randomly selected soil subsamples (Ap horizon, depth: 0 – 10 cm) on 32 m² (*n* = 4) from the plots without fertilizer and without MP using a soil core sampler (cross-sectional area: 9.53 cm²) before the start of the experiment. To analyze MP particles added to the field and soil biological variables, before setup and 1 month (M1), 5 months (M5), and 17 months (M17) after setup, 8 subsamples were taken from a 4 m² sampling square in the center of each plot (Ap horizon, depth: 0 – 10 cm) and pooled into composite samples of approximately 1 kg for each time point. Since the soil sampled in this way contained very few MP particles > 0.5 mm at M1 and M5, we additionally sampled an area of 900 cm² per plot using a spade at the end of the experiment (M17).

Soil samples for soil biological analyses were stored at –20 °C until analysis.

Microplastics analyses

To characterize the background contamination of the arable soil with MP and to investigate the fate of added MP particles, MP were extracted and measured according to *Möller* et al. (2022).

In brief, soil samples were freeze-dried and sieved to 0.5 mm. All further analyses were done with aliquots of 250 g soil.

MP > 0.5 mm were collected with tweezers and analyzed by attenuated total reflection – Fourier transform infrared (ATR-FTIR) spectrometry (spectrometer: Alpha ATR unit, Bruker 27; equipped with a diamond crystal for measurements). Spectra were taken from 4000 to 400 cm^{-1} (resolution 8 cm^{-1}, 16 accumulated scans, Software OPUS 7.5). Particles were identified by comparing the measured spectra against standard spectra from an in-house database described previously (*Löder* et al., 2015) and the database provided by the manufacturer of the instrument (Bruker Optik GmbH, Leipzig, Germany). An incident light microscope (microscope, Nikon SMZ 754T; digital camera, DS-Fi2; camera control unit, DS-U3; software, NIS Elements D) was used for visual documentation and size estimation of all synthetic plastic particles identified by ATR-FTIR.

Soil samples taken from the 900 cm^2 areas at M17 (corresponding to approximately 10 L soil) were analyzed in their entirety to detect large particles > 0.5 mm. To this end, the soil samples were partitioned into 20 Fido jars (Bormioli Rocco, Fidenza, Italy; capacity 3 L each) and suspended with 2.5 L of water. The diluted samples were sieved at 2 mm and the retained particles were collected with tweezers (fraction > 2 mm). All material < 2 mm was sieved at 0.5 mm mesh size, and the retained particles were again collected with tweezers (fraction 0.5 – 2 mm).

According to *Möller* et al. (2022), MP < 0.5 mm were extracted via density separation with a zinc chloride brine (ρ = 1.8 g cm^{-1}) and an enzymatic-oxidative purification step (Löder et al., 2017). Particles were then transferred onto an aluminum oxide sample carrier and analyzed by chemical imaging via Focal Plane Array-based µ-Fourier-transform infrared (µ-FTIR) spectroscopy (*Löder* et al., 2015). Identification of MP in the large chemical imaging data sets was performed with the help of an automated software solution based on Random Decision Forest Classifiers (*Hufnagl* et al., 2022). For quality control the results of the automated MP classification was checked by trained experts. We only analyzed the samples from plots without organic fertilizers, i.e., the samples from 12 out of 36 plots (see Fig. 7-2), at M1 and M17 as well as the MP-soil mixtures that were added to the plots with MP treatment (in total: 12 + 12 + 8 = 32 samples). We had to limit the number of analyzed samples due to the extensive and time-consuming extraction and purification procedure (*Möller* et al., 2022). In addition, the high organic matter content of compost and digestate interferes with the treatment of the samples. Thus, these samples could not be analyzed. Due to high numbers of MP particles,

deviating from the above-mentioned protocol, for the initial MP-soil-mixtures that were added to the field, four subsamples of 5 g each were analyzed.

We calculated the initial MP concentrations in soil at the start of the field experiment (MP_{start}), assuming that the applied MP-soil-mixtures were homogeneously mixed within the top 10 cm of soil (Equation 1)

$$c_{MP,i} = \frac{m_{mix} \cdot c_{mix}}{(d \cdot \rho_B)} \quad (1)$$

where $c_{MP,i}$ is the initial MP concentration in the soil of the field experiment (particles kg^{-1}), m_{mix} is the mass of applied MP-soil-mixtures per area (0.294 kg m^{-2}), c_{mix} is the measured MP concentration of the MP-soil-mixtures (particles kg^{-1}), d is the depth of the soil layer (10 cm), and ρ_B is the bulk density of top soil (1400 kg m^{-3}).

Since soil samples were separated into two fractions due to sieving of 0.5 mm and these two fractions were analyzed differently as described above, we excluded particles > 0.5 mm in the small fraction (5.1 – 38.5 %) and particles < 0.5 mm in the large fraction (0 – 2.1 %), respectively (Table S7-1). Due to sieving of MP to 2 mm before use in our study, particles > 2 mm were filtered from datasets (this applied only to MP-soil-mixtures).

We derived particle size distributions of LDPE and PLA/PBAT particles as initially added to the soil based on MP particles detected in MP-soil-mixtures (Fig. S7-1). The median size of LDPE particles in the small and large fractions were 186 µm and 1092 µm, respectively (Fig. S7-1**)**. The median size of PLA/PBAT particles in the small and large fractions were 200 and 1013 µm, respectively (Fig. S7-1).

Soil microbiological indicators of carbon cycling

To assess effects of MP and organic fertilizers on the soil microbial abundance and activity, we used microbial biomass C and activities of enzymes involved in C cycling as soil microbiological indicators. These were measured before setup, 1 month (M1), 5 months (M5), and 17 months (M17) after the setup of the experiment.

Microbial biomass C (C_{mic}) and nitrogen (N_{mic}) were quantified via chloroform fumigation extraction according to *Vance* et al. (1987). For a description of the method, we refer to *Blöcker* et al. (2020).

We analyzed the activity of enzymes that catalyze the degradation of organic substrates of different complexities: we considered β-glucosidase, N-acetyl-glucosaminidase, ß-xylosidase, and phenoloxidase as indicators of the degradation of the polymers cellulose, chitin, xylan (hemicellulose), and lignin. In addition, we analyzed the activity of lipase because of its

possible involvement in the depolymerisation of PLA/PBAT. The activities of ß-glucosidase, β-xylosidase, N-acetyl-glucosaminidase, and lipase were measured using microplate assays with fluorogenic substrates (*German* et al., 2011; *Marx* et al., 2001; *Cooper* and *Morgan*, 1981). Lipase activity was determined based on an adapted protocol from *Cooper* and *Morgan* (1981). Substrates and standards were purchased from Sigma-Aldrich (St. Louis, MO, United States). Standard stock solutions of 5 mM 4-methylumbelliferyl (MUF, M1381) were obtained by dissolving MUF in methanol and deionized water (1:1). Standard working solutions (10 µM MUF) were prepared in 0.1 M Tris-HCl buffer pH 6.8 (lipases) or MES buffer pH 6.1 (ß-glucosidase, ß-xylosidase, N-acetyl-glucosaminidase). For each soil sample, we prepared a standard curve with concentrations of 0, 0.5, 1, 2.5, 4, 6 µM MUF in soil suspension aliquots and buffer. Lipase substrate stock solutions (10 mM) were obtained by dissolving the substrates 4-methylumbelliferyl heptanoate (M2514) in dimethyl sulfoxide (D8418). Working solutions (1 mM) were prepared by adding sterile 0.1 M Tris-HCl buffer pH 6.8. Substrate solutions of ß-glucosidase, ß-xylosidase, and N-acetyl-glucosaminidase were prepared and analyzed as outlined in *Kramer* et al. (2013).

Soil phenoloxidase activity was analyzed based on *Floch* et al. (2007) using 2,2′-azinobis-(-3 ethylbenzothiazoline-6-sulfononic acid) diammonium salt (ABTS, A1888, Sigma-Aldrich, St. Louis, MO, United States) as the substrate. ABTS can be oxidized by phenoloxidases to the blue-green-colored $ABTS^{\cdot+}$ radical cation, which can be measured photometrically (*Floch* et al., 2007; *Koleva* et al., 2001). ABTS stock and working solutions (10 and 2 mM) were prepared in modified universal buffer (MUB) pH 4. MUB was prepared by dissolving tris(hydroxymethyl)aminomethane (4855.2, Carl Roth GmbH + Co. KG, Karslruhe, Germany), maleic acid, citric acid, and boric acid in deionized H_2O and adjusting to pH 4 with 1M NaOH (*Floch* et al., 2007).

ABTS converts to $ABTS^{\cdot+}$ by reacting with potassium persulfate ($K_2S_2O_8$, 1.05091, Merck KGaA, Darmstadt, Germany) in a ratio of 2:1 (*Koleva* et al., 2001). As the standard, a $ABTS^{\cdot+}$ solution was prepared. First, 8 mM phosphate-buffered saline (PBS) pH 7.4 was prepared according to *Koleva* et al. (2001). Then, a 50 mM $K_2S_2O_8$ solution was prepared in PBS. To obtain the final 1 mM $ABTS^{\cdot+}$ standard solution, 1 mL from the 10 mM ABTS stock solution was thourougly mixed with 8.89 mL of the PBS solution and 0.11 mL of the $K_2S_2O_8$ solution. Mixing in this manner yields a small excess of $K_2S_2O_8$ in the standard solution, which promotes the conversion from ABTS to $ABTS^{\cdot+}$. Next, the $ABTS^{\cdot+}$ standard solution was incubated in the dark at room temperature overnight. The conversion of ABTS to $ABTS^{\cdot+}$ was verified by the presence of a clear extinction maximum at 414 nm using a photometer (Synergy HTX multi-

mode reader, Bio-Tek Instruments Inc., Winooski, VT, USA). On each microplate, calibration curves were prepared with final concentrations of 0, 20, 40, 60, 80, and 100 µM $ABTS^{\cdot+}$ that were prepared in MUB pH 4.

To measure soil phenoloxidase activity, 0.4 g fresh soil was suspended in 50 mL deionized H_2O and dispersed through ultrasonication for 2 min at 50 J s^{-1}. Per sample, in triplicate, 100 µl soil suspension and 100 µL MUB pH 4 were pipetted onto one microwell. Then, 50 µl ABTS working solution was added. Also, controls without substrate were employed in triplicate (100 µl soil suspension and 150 µL MUB pH 4). The microplates were preincubated at 30 °C for 5 min, before absorbance was read by the photometer at a wavelength of 414 nm over 30 min at 3 min - intervals.

Crop yields

Silage maize and summer barley were harvested in September of the first year (4 months after setup) and in August of the second year of the experiment (15 months after setup), respectively (Fig. 7-1b**)**.

To determine the biomass of the silage maize (*Zea mays*), we removed every second plant by cutting it 1 cm above its root system. We determined maize plant dry matter biomass (including cobs) after chopping the plants and drying them at 60 °C and 110 °C (for 3 days each). Two-step drying is common practice at the research station to accelerate drying to mass constancy at 110 °C. We then multiplied mean silage maize biomass per plot by the number of plants per plot to obtain silage maize biomass yield per plot. Grain yield of summer barley (*Hordeum vulgare*) was determined from an area of 12 m² (1.5 m * 8 m) per plot and grains were sampled using a plot threshing machine. Crop yields were converted to t ha^{-1}.

Data analyses

All data analyses and figures were carried out using the statistical software R 4.0.2 (*R Core Team*, 2020). In addition to packages explicitly mentioned in this section, we used the following extensions: broom.mixed 0.2.6 (*Bolker* and *Robinson*, 2020), broom 0.7.0 (*Robinson* et al., 2020), flextable 0.5.10 (*Gohel*, 2020), patchwork 1.0.1 (*Pedersen*, 2020), scales 1.1.1 (*Wickham* and *Seidel*, 2020), and tidyverse 1.3.0 (*Wickham* et al., 2019).

The MP background concentrations (before setup) and concentrations of MP particles > 0.5 mm were evaluated only descriptively because there were too few data for inferential statistical analysis. For particles < 0.5 mm, differences in particle number between MP_{start}, M1, and M17 were tested using a linear mixed effects model with particle number as dependent variable, and

timepoint (MP_{start}, M1, and M17) as the explanatory variable, while accounting for a random effect for plot (ID). Tukey contrasts were computed using functions from the emmeans 1.5.0 package (*Lenth*, 2020). Particle size distributions of particles < 0.5 mm were compared by plotting empirical cumulative density functions and using the Kolmogorov-Smirnov test (ks.test), to test whether the MP particles in MP_{start}, M1, and M17, originated from the same distribution (*van Schothorst* et al., 2021). Empirical cumulative density functions were calculated based on pooled samples per treatment group (n = 4).

Crop yields were evaluated using a linear model with the crossed factors plastic type and fertilizer and accounting for a block effect. Soil enzyme activities, C_{mic}, and N_{mic} data were analyzed by means of linear mixed effects models. Therefore, the linear model used for crop yield data was extended by the initial state of the variable of interest as covariate to account for the field variability (Value_TMinus1). We integrated the repeated measures factor timepoint (i.e., M1, M5, and M17) by crossing with treatment structure, and accounted for a block and block - timepoint interaction effect as well as a random effect for the randomization unit (i.e., plot) (*Piepho* et al., 2004). The models were fitted to the data using functions from base R and the package lme4 1.1-23 (*Bates* et al., 2015). We used ANOVAs in the case of linear mixed effects models with the Kenward-Rogers approximation for the degrees of freedom using functions from the lmerTest 3.1-2 package (*Kenward* and *Roger*, 1997; *Kuznetsova* et al., 2017) to identify significant effects ($p < 0.05$) and subsequently compared estimated marginal means. If an interaction with timepoint was significant, we evaluated simple contrasts per timepoint level.

Model assumptions, i.e., variance homogeneity and normal distribution of the residuals, were checked visually and considered met for all variables except for N-acetyl-glucosaminidase activity, for which the model assumptions were met after log-transformation.

7.4 Results

Background loading of microplastics in the arable soil

The arable soil had a MP loading with nine different polymer types at a background concentration of 296 ± 110 (mean ± standard error) particles kg^{-1}. Polypropylene (PP, 108 ± 36 particles kg^{-1}), polystyrene (PS, 76 ± 34 particles kg^{-1}), and polyethylene (PE, 60 ± 25 particles kg^{-1}) were the most abundant polymers and were found in all analyzed samples (Fig. 7-3a). Other MP were polyacrylonitrile, polyethylene terephthalate, polyvinyl chloride, polybutylene terephthalate, ethylene-vinyl acetate, and polysulfone.

PS particles were smallest with a median particle size of 60 µm (Fig. 7-3b). PP and PE particles had median sizes of 156 µm and 146 µm, respectively. While the particle size distribution of PS MP was significantly shifted to lower particle lengths compared to PP ($p = 0.014$), the particle size distribution of PE MP was similar to that of PP and PS MP ($p = 0.187$ and $p = 0.188$).

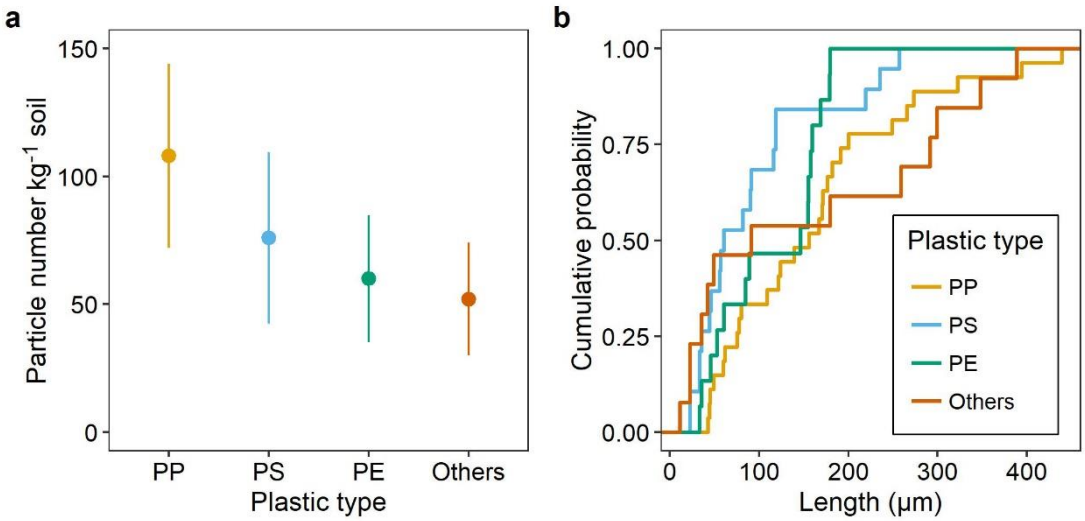

Fig. 7-3. a Particle numbers of PP, PS, PE, and other polymers < 0.5 mm. Data are presented as means and standard errors (error bars) ($n = 4$). **b** Empirical cumulative distribution function of pooled samples for PE (15 particles), PS (19 particles), PP (27 particles), and others (13 particles). Other MP were polyacrylonitrile, polyethylene terephthalate, polyvinyl chloride, polybutylene terephthalate, ethylene-vinyl acetate, and polysulfone.

Fate of added microplastics < 0.5 mm in soil

At the start, soil amended with LDPE and PLA/PBAT contained 1003 LDPE kg^{-1} and 134 PLA/PBAT particles kg^{-1} of MP < 0.5 mm (MP_{start}, Fig. 7-4a). After one month (M1), we detected on average 419 fewer LDPE particles kg^{-1} than at MP_{start} (not significant, $t_6 = –2.7$, $p = 0.082$). The mean number of LDPE particles 17 months after MP addition (M17) and PLA/PBAT particles at M1 and M17 did not differ significantly from MP_{start} (Table S7-2, Fig. 7-4a). The particle size distribution of LDPE and PLA/PBAT MP at M1 and M17 did not differ from MP_{start} (Fig. 7-4b).

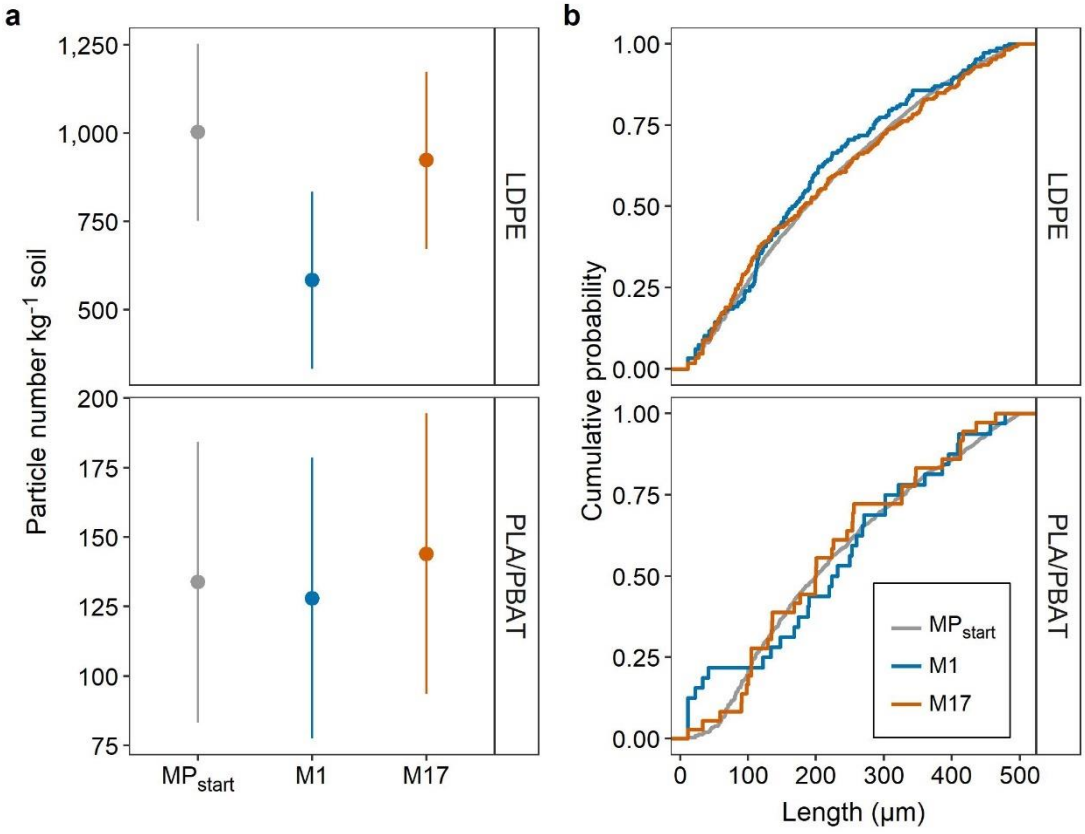

Fig. 7-4. a Particle numbers of LDPE and PLA/PBAT particles after application of MP-soil-mixtures as initially added to the plots (MP_{start}), after one month (M1), and after 17 months (M17). Data are presented as estimated marginal means with lower and upper 95 % confidence intervals (error bars) (*n* = 4). Note that y-axis scales for LDPE and PLA/PBAT differ from one another. **b** Empirical cumulative density functions of number of LDPE and PLA/PBAT particles in MP-mixtures as initially added to the plots (MP_{start}), at M1, and at M17, pooled by plastic type.

Fate of added microplastics > 0.5 mm in soil

We found a total of 57 particles > 0.5 mm (27 varnish, 13 PE, 16 PLA/PBAT, and 1 PP) at the final sampling (M17), in all soil samples taken together (n = 36). PLA/PBAT and LDPE particles (up to 2) were detected in soil samples from only two (PLA/PBAT) and three plots (LDPE) without fertilizer treatment, respectively. Due to this low recovery, a quantitative comparison of particles > 0.5 mm with MP_{start} was not possible. PLA/PBAT particles occurred only in soil samples from plots where PLA/PBAT had been added (Fig. 7-5a – c). All PLA/PBAT found looked similar (white and irregularly shaped) (Fig. 7-5 a – c) and like the originally added particles (Fig. S7-2).

However, PE particles (Fig. 7-5d – f) occurred not only in soil samples of plots, where PE had been added. They also had different shapes including plastic film residues (Fig. 7-5d), fibers (Fig. 7-5e), or irregularly shaped pieces (Fig. 7-5f). PE particles found were distinct from the

initially added PE particles **(**Fig. S7-2d – f**).** All varnish particles were of the same type (Fig. 7-5g – i).

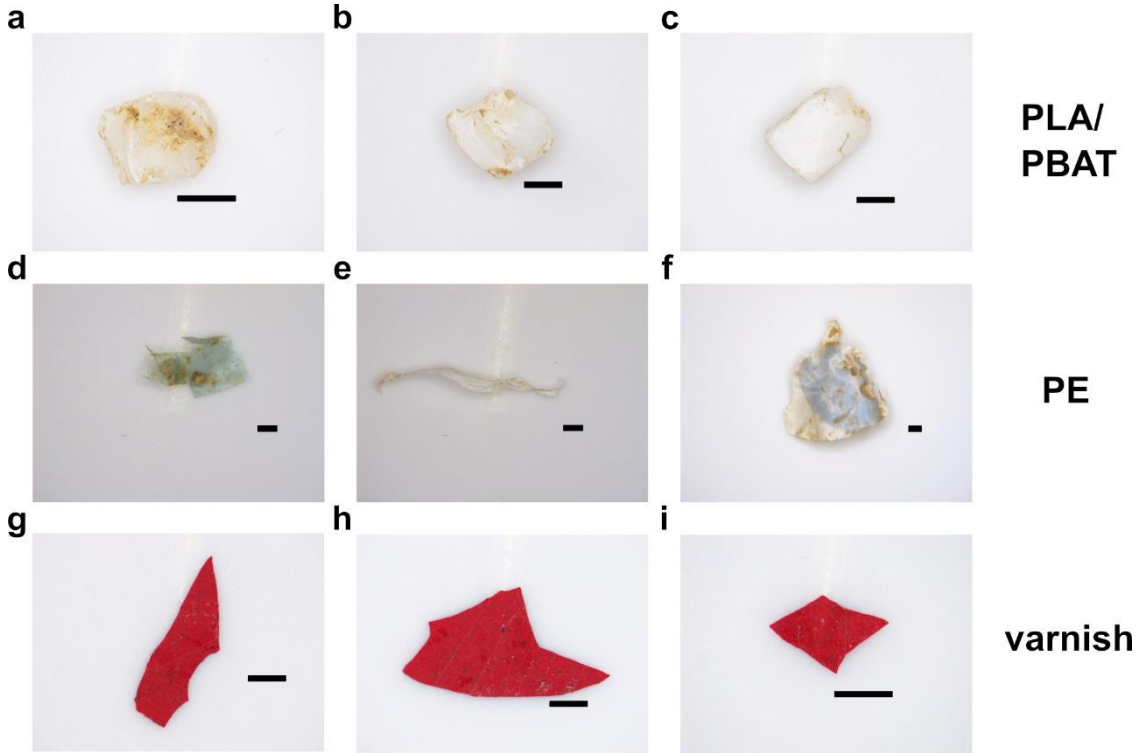

Fig. 7-5. Representative microscopic images of MP > 0.5 mm: **a** – **c** PLA/PBAT, **d** – **f** PE, and **g** – **i** varnish found after 17 months (M17). The scale bars indicate a length of 1 mm.

Soil microbiological indicators of carbon cycling and crop yields

We investigated the effects of adding 2 g MP m^{-2} on soil microbial abundance and activity related to C cycling and crop yields based on soil microbiological indicators (C_{mic}, N_{mic}, and activities of C cycling enzymes), biomass of silage maize, and grain yield of summer barley. Overall, MP from LDPE and PLA/PBAT did not cause changes of the soil microbiological indicators at one, five, and 17 months after MP addition, or in crop yields compared to MP-free soil (Fig. 7-6, Fig. S7-3, Fig. S7-4, Table S7-3, Table S7-4). The exception was LDPE at M5, which reduced N_{mic} significantly by 36 % compared to the MP-free soil (Fig. S7-4a, Table S7-5).

No combined effects of MP with organic fertilizers were detected, but amendment of soil with composts and digestates affected the activity of C cycling enzymes in soil (Fig. 7-6b, Fig. S7-3, Table S7-3, Table S7-5, Table S7-6). Lipase activities responded to the addition of compost (M1 and M5) and digestate (M5) significantly increasing from 37 – 62 % compared to fertilizer-

free soil (Fig. 7-6b**,** Table S7-5). ß-xylosidase showed significantly enhanced activity in soil amended with digestate in comparison to the fertilizer-free soil at M5 (+ 60 %) and M17 (+23 %) (Fig. S7-3b, Table S7-5). Both ß-xylosidase and ß-glucosidase activities increased by 47 % in response to compost addition at M5 compared to the fertilizer-free soil, but statistical uncertainties were large for ß-xylosidase (p = 0.061) (Fig. S7-3a & b**,** Table S7-5). Compared to non-fertilized soil, N-acetyl-glucosaminidase activities increased 59 % (significant) after digestate addition at M5 (Fig. S7-3c, Table S7-6)**.**

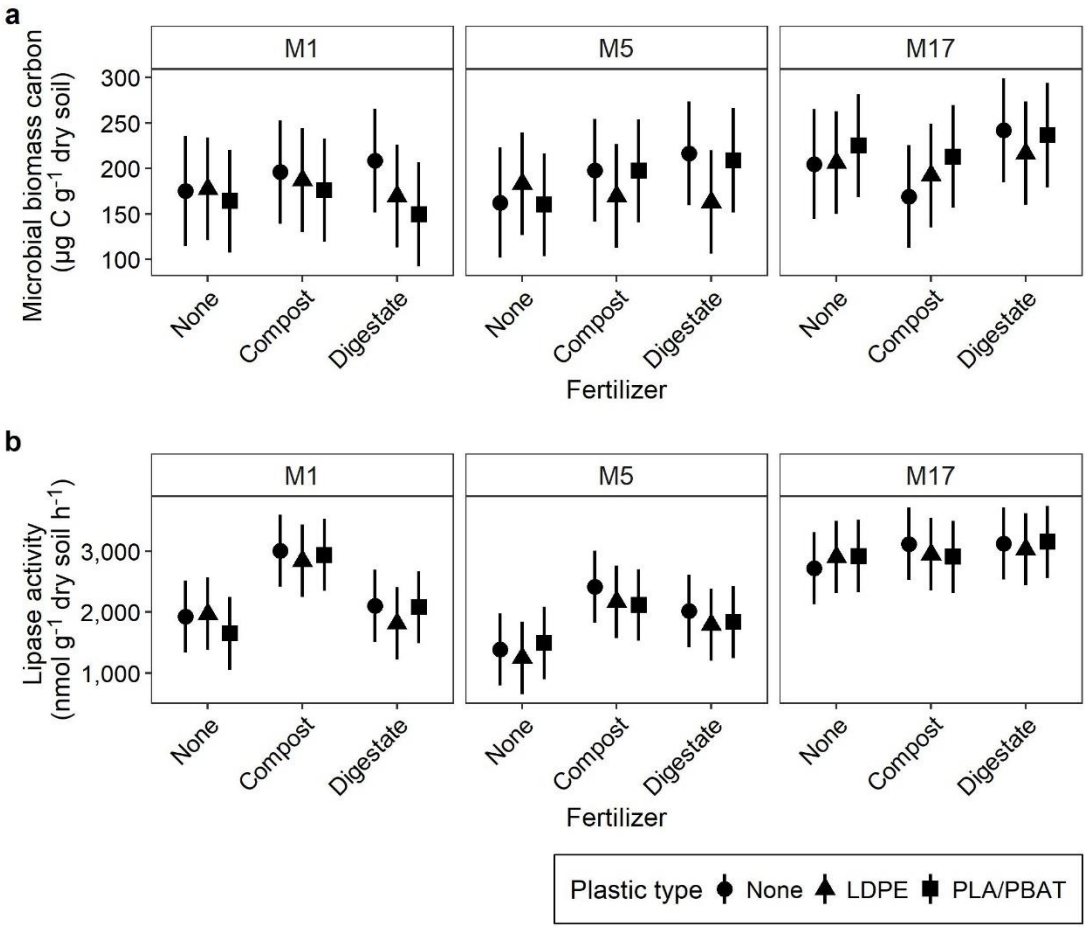

Fig. 7-6. a Microbial biomass C and **b** lipase activity as a function of MP and organic fertilizers one month (M1), five (M5) and 17 months (M17) after the addition of 2 g MP m^{-2}. Data are presented as estimated marginal means (n = 4) with lower and upper 95 % confidence intervals (error bars).

After 17 months, the activities of ß-xylosidase, N-acetyl-glucosaminidase and ß-glucosidase were significantly higher in the soil amended with digestate compared to compost (Fig. S7-3 a – c, Table S7-5). Strikingly, this coincided with increased N_{mic} in the soil enriched with digestate compared to compost at M17 (+ 22 %, p = 0.026) (Fig. S7-4 a**,** Table S7-5).

Independent of timepoint, phenoloxidase activity was 16.6 % higher in soil amended with digestate in comparison to fertilizer-free soil (Fig. S7-3d). However, statistical uncertainties were large (p = 0.069) (Table S7-7).

Biomass yields of silage maize (mean and standard error: 19.70 ± 0.48 t ha^{-1}) were not significantly higher on soil amended with compost and digestate in comparison to non-fertilized soil (Fig. S7-4b, Table S7-4**)**. However, grain yield of spring barley (estimated marginal mean: 6.95 t ha^{-1}) was larger (significantly) on soil amended with digestate compared to compost (6.31 t ha^{-1}) and larger (though not significantly) than on non-fertilized soil (6.46 t ha^{-1}) (Fig. S7-4c**,** Table S7-7).

7.5 Discussion

The arable soil was loaded with diverse microplastics types

The arable soil in our study contained 296 ± 110 (mean ± standard error) MP particles < 0.5 mm kg^{-1} as background concentration. This concentration was lower than estimates for arable soils amended with compost (888 ± 500 particles kg^{-1} soil, *van Schothorst* et al., 2021), sewage sludge (930 ± 740 particles kg^{-1} soil for low-density plastics and 1100 ± 570 particles kg^{-1} for high-density plastics; *van den Berg* et al., 2020), or plastic mulch (18,760 particles kg^{-1} soil; *G.S. Zhang* and *Liu*, 2018).

The most common plastic types found in our soil were PP > PS > PE. These are among the most economically important polymers and are also those that have previously been most frequently detected in soil (*PlasticsEurope*, 2019; *X. Zhang* et al., 2021). In accordance with our results, *Piehl* et al. (2018) identified PP, PS, PE as the most abundant MP particles (> 1 mm) in a conventionally managed field that had not been amended with organic fertilizers, sewage sludges, where plastic mulches had been applied. Since the input of MP via the latter sources can be excluded in our study, the recovered MP presumably entered the soil by littering and atmospheric deposition (*Allen* et al., 2019; *Dris* et al., 2016; *Kernchen* et al., 2022; *Scheurer* and *Bigalke*, 2018;). The relatively high number of extracted varnish particles (Fig. 7-5) suggest that abrasion of protective coatings from agricultural machinery could be an important source of MP in arable soils (Fig. S7-5).

We found that more than 75% of the PP, PS, and PE particles were smaller than 0.2 mm (PS: < 117 µm, PE: < 159 µm, PP: < 196 µm), consistent with previous results from *J. Wang* et al. (2021). The current detection limit is 10 µm (*Möller* et al., 2020); we expect, therefore, that smaller particles occur even more frequently. This could have dramatic consequences for soil organisms because particles < 10 µm can be ingested by key member species of the soil food

web such as nematodes, resulting in intestinal damage and neurotoxicity (*Fueser* et al., 2019; *Lei* et al., 2018; *Schöpfer* et al., 2020). PS particles in particular pose a risk to soil animals; these were the smallest in our study (median of 60 µm). However, concentrations of small MP down to nanometer sizes are currently undetectable due to restrictions of analytical methods (*Möller* et al., 2020). Further progress in MP analytics is needed to better assess potential threats of small MP to soil organisms and their functions.

We can confidently state that the PLA/PBAT particles > 0.5 mm we found at the last sampling of the experiment (M17) were the particles we had added. We found these exclusively in the PLA/PBAT treated plots but with no finds in the corresponding background loading. All PLA/PBAT particles looked similar and resembled the original particles. In contrast, we cannot rule out that a significant portion of the PE particles we found were part of the background loading. For one thing, LDPE particles also occurred in plots to which no LDPE had been added, and for another, the PE particles found had various shapes (Fig. 7-5) and differed from the originally added LDPE particles (Fig. S7-2).

At the last sampling, we found only very few particles > 0.5 mm. We can exclude the possibility that the particles had been fragmented (with the exception of the fragmentation < 0.01 mm, which we could not detect with our method) because this should have been detected via a clear shift in the size distribution of the particles < 0.5 mm. The low recovery, we suggest, could be due to the possibility that the amount of soil or area sampled was insufficient or that the methodology for analyzing these large particles needs further development. Methodological limitations apply especially to the LDPE particles, which had a more fibrous shape than the predominantly irregularly shaped PLA/PBAT particles. The LDPE particles may have been more prone to fall through the sieve during MP analysis in wet sieving. It is also possible that a significant proportion of large particles were transported vertically or horizontally. A recent study provides evidence for horizontal transport of MP (irregularly shaped polymethyl methacrylate particles with a mean length of 1215 µm), which occurred along preferential pathways dictated by the micro- and macro-relief of the soil surface (*Laermanns* et al., 2021). However, more studies on the transport (including vertical transport) of particles in the field will be required to test our assumption.

Microplastics persisted in the arable soil

Both tested polymers persisted in the soil of the field experiment over 17 months. The number of added LDPE particles < 0.5 mm (584 – 1003 particles kg^{-1}, Fig. 7-4) in our study roughly represents the LDPE accumulation that can be expected after 7 – 20 years of compost

accumulation (*van Schothorst* et al., 2021). PE is highly resistant to microbial degradation in soil due to its large molecular size, lack of functional groups, and high hydrophobicity (*Albertsson*, 1978; *Krueger* et al., 2015), which explains the unaltered particle size distribution compared to the initial particles, indicating a lack of fragmentation in the studied soil. Surprisingly, *S. Zhang* et al. (2020) found that fertilization with N and phosphorous stimulates the fragmentation of LDPE. According to the authors, LDPE fragmentation was triggered by increased soil microbial diversity and abundance. This behavior and its mechanisms need to be confirmed by further studies.

Contrary to our expectations, we recovered the same number of PLA/PBAT particles < 0.5 mm as initially added to the soil, most likely due to the lack of biodegradation (Fig. 7-4). The few existing studies on the persistence of films of PLA, PBAT, and PLA/PBAT blends in soil under field conditions demonstrate their low biodegradability (*Liao* and *Chen*, 2021; *Rudnik* and *Briassoulis*, 2011; *Sintim* et al., 2020). PLA exhibited changes in mechanical properties after 11 months in a Mediterranean soil but was visually poorly disintegrated (Rudnik & Briassoulis, 2011). In another study, mass loss of 1 – 8 % and 1 – 7 % were observed for PLA and PBAT, respectively, after 6 months, whereas a PBAT/ PLA blend (90/10 % w/w) showed no significant degradation (*Liao & Chen*, 2021). A lower degradability of PLA/PBAT (75/25 % w/w) blend compared to the sole polymers was also observed in a laboratory study (*Palsikowski* et al., 2018). While 21 % of the PBAT- and 16 % of PLA-C were mineralized, only 10 % of PLA/PBAT-C were mineralized after 180 days in soil. *Liao* and *Chen* (2021) attributed the poor degradation of the blend in their study to the blending of PLA with PBAT; blending would change physical properties and increase hydrophobicity, thus impeding microbial colonization and microbial degradation. This could explain, why no fragmentation of PLA/PBAT was observed in our study.

Based on our results, non-biologically pretreated PLA/PBAT particles are likely to accumulate in the soil under field conditions, given the highly variable climatic conditions with extremes such as cold and drought that may slow the biodegradation of PLA/PBAT.

Microplastics did not affect soil microbial biomass, enzyme activities, and crop yields

We did not find any effect of LDPE on soil microbiological indicators of C cycling, likely due to its inert nature (*Restrepo-Flórez* et al., 2014). However, we found an effect of LDPE on N_{mic} (Fig. S7-4), but this occurred only sporadically (at one timepoint) and the measurement uncertainties were large (Table S7-5). In line with our results, *Lin* et al. (2020) did not observe

significant changes in soil microbial biomass C and microbial community composition due to the addition of LDPE at concentrations 5, 10, 15 g m^{-2} (corresponding to 11,361, 23,789, and 39,172 particles kg^{-1}). In a recent field study, no effects of LDPE-MP on microbial abundance and composition were detected even at extremely high application rates up to 1000 g MP m^{-2} (*Brown* et al., 2022). However, *Lin* et al. (2020) found substantial increases in C cycling enzymes such as α-glucosidase and ß-glucosidase at all concentration levels between 36 and 86 %, and an increase in L-leucine aminopeptidase, a N cycling enzyme, by 83 – 116 %. They explained the enhanced enzyme activities by greater water availability due to a MP-induced increase of water holding capacity, which would positively influence enzyme activities. Compared to *Lin* et al. (2020), in our study we used LDPE particles at a much lower concentration of 2 g m^{-2} MP (584 – 1003 LDPE particles kg^{-1}), and larger LDPE particles (Fig. S7-1, 90th percentile of particles < 0.5 mm and > 0.5 mm of 430 and 1619 µm, respectively, compared to a 90th percentile of 68 µm in their study). Accordingly, particles in our study had a lower specific surface area with less potential to affect soil physical properties including water holding capacity (*Ng* et al., 2018).

Contrary to our expectations, the addition of PLA/PBAT particles did not affect any of the soil microbiological indicators of C cycling. This was likely due to the lack of biodegradation of PLA/PBAT particles (see section "Microplastics persisted in the arable soil") in soil and to the fact that soil microorganisms were apparently not able to use the added PLA/PBAT blend as a C source. In another study, PBAT/PLA MP affected soil C and N pools (*Meng* et al., 2022). For instance, there were significantly higher dissolved organic C and N due to addition of 2 and 2.5 % PBAT/PLA MP additions in comparison to the control. Again, the lower concentration of PLA/PBAT particles in our study could explain why we did not detect changes in soil microbiological indicators of C cycling.

We verified previous studies in which compost and digestate led to a stimulation of enzyme activities (*Alburquerque* et al., 2012; *Crecchio* et al., 2004; *Vinhal-Freitas* et al., 2010). Depending on the quality of the organic fertilizers, we found slightly different temporal patterns of degradation of high molecular weight organic compounds. The increased lipase activities in fertilized soil after one and five months of addition reflected the rapid breakdown of fats and oils contained in compost and digestate into free fatty acids, diacylglycerols, monoglycerols, and glycerol (*Hanc* et al., 2021). The more pronounced increase due to compost compared to digestate addition indicates a higher lipid content in compost than in digestate. Breakdown of other compost- and digestate-derived polymers (hemicellulose, cellulose, and chitin) were induced at a later timepoint. For example, the degradation of chitins in soil fertilized with

digestate as well as the degradation of cellulose in compost-amended soil were only evident five months after addition. The degradation of hemicellulose derived from amendments was still visible after 17 months. Since we did not find any differences in microbial biomass under the two organic amendments, the observed increase in activities was likely due to higher enzyme production of already present microorganisms.

Crop yield, i.e., silage maize biomass and grain yield of summer barley, was not affected by MP addition in our study. Direct effects due to uptake and accumulation in plants have been observed for MP < 2 µm (*Mateos-Cárdenas* et al., 2021). Uptake by plants was unlikely in our study since MP were too large for uptake by plants. While additional mechanisms of MP effects on plant biomass remain unclear, changes in soil structure, bulk density, improved aeration and microporosity, as well as rooting and nutrient immobilization, are discussed as possible results of both negative and positive effects of MP on plant biomass (*Boots* et al., 2019; *Lozano* et al., 2021; *Mateos-Cárdenas* et al., 2021; *Qi* et al., 2018; *Rillig* et al., 2019). Such indirect effects are again likely to occur if MP concentrations exceed certain thresholds, which may be the case in fields with plastic mulch and sewage slugde application where MP loadings are particularly high (*Büks* and *Kaupenjohann*, 2020; *G.S. Zhang* and *Liu*, 2018; *D. Zhang* et al., 2020). However, *Brown* et al. (2022) did not observe growth and yield reductions of wheat plants even with loads of LDPE-MP > 100 g m^{-2}. While these results, as in our case, indicate that MP might not pose a risk with respect to plant growth, this should be confirmed by investigations of other sites (soil type, climate) as well as plant species and MP types. Nevertheless, for fields with lower MP concentrations, such as in our study, no negative effects of MP on plant biomass can be expected.

7.6 Conclusions

Our results highlight that diverse MP can be found in arable soils even without agricultural practices such as organic fertilization, sewage sludge addition, or plastic mulching. This indicates that there are significant diffuse MP inputs into soils through atmospheric deposition, littering, and, to our knowledge noted for the first time, due to the abrasion of coatings of agricultural machinery. In particular, small MP particles < 0.2 mm were frequently found in the soil. Soil organisms can ingest such particles with to-date unknown long-term environmental risks. There remains much uncertainty regarding concentrations of small MP < 0.01 mm and nanoparticles, and methods for their detection in soil are needed.

We provide evidence that conventional as well as biodegradable MP can persist and accumulate in soil under field conditions. Current MP loadings in arable soil under agricultural practices

such as amendment with organic fertilizers have no detectable immediate negative consequences neither on soil microbial abundance and activity related to C cycling, nor on crop yields. However, due to regular MP inputs from diffuse sources and from organic fertilizers and sewage sludge contaminated with MP, as well as high persistence of many polymers, long-term effects of MP on soil microbial abundance and activities related to C and nutrient cycling cannot be excluded. Additional long-term field studies examining different soil types and polymers will be crucial to assess the risks of environmental threats of MP to functions of agricultural soils.

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7.8 Data Availability Statement

The data that support the findings of this study are openly available on Mendeley at http://doi.org/10.17632/8chdw8vgw9.2.

7.9 References

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7.10 Supporting information

Supporting information is available at:

https://onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1002%2Fjpln.202200062&file=jpln202200062-sup-0001-SuppMat.pdf

Tables

Table S7-1. Number and proportion of particles < 0.5 mm and > 0.5 mm by sample type and analysis (µFTIR, ATR-FTIR). Particles that were removed from the small fraction (µFTIR analysis) and large fraction (ATR-FTIR analysis) datasets are shown in grey. M1 and M17 stand for one month and 17 months after setup of the experiment.

Analysis	Sample type	Particle numbers (absolute)		Proportion of particles (%)	
		< 0.5 mm	> 0.5 mm	< 0.5 mm	> 0.5 mm
small fraction (µFTIR-ATR)	Background contamination (all MP types)	74	4	94.9	5.1
	LDPE-soil-mixture (only PE)	4774	1246	79.3	20.7
	Soil from LDPE plots at M1 (only PE)	146	34	81.1	18.9
	Soil from LDPE plots at M17 months (only PE)	231	36	86.5	13.5
	PLA/PBAT-soil-mixture (only PLA/PBAT)	1274	475	72.8	27.2
	Soil from PLA/PBAT plots at M1 (only PLA/PBAT)	32	20	61.5	38.5
	Soil from PLA/PBAT plots at M17 (only PLA/PBAT)	36	5	87.8	12.2
large fraction (ATR)	LDPE-soil-mixture (only PE)	27	1249	2.1	97.9
	PLA/PBAT-soil-mixture (only PLA/PBAT)	1	952	0.1	99.9
	Soil from all plots at M17 (all polymer types)	0	57	0	100

Table S7-2. Pairwise contrasts of LDPE and PLA/PBAT particle number of MP_{start}, M1 and M17 separate for MP type. Lower and upper 95 % confidence intervals (CI low, CI up), *t* statistics, *df* degrees of freedom and *p* values. Numbers in bold indicate *p* values < 0.05.

MP type	Contrast	Difference	CI low	CI up	*t*	*df*	*p*
LDPE	M1 – MP_{start}	-419	-900	62	-2.67	6	0.082
	M17 – MP_{start}	-79	-560	402	-0.50	6	0.872
	M17 – M1	340	-141	821	2.17	6	0.156
PLA/PBAT	M1 – MP_{start}	-6	-100	89	-0.19	6	0.981
	M17 – MP_{start}	10	-84	105	0.33	6	0.942
	M17 – M1	16	-78	110	0.52	6	0.865

Table S7-3. ANOVA tables with variable (Var.), numerator degrees of freedom (Num *df*), denominator degrees of freedom (Den *df*), sum of squares (sumsq), mean sum of squares (meansq), *F* and *p* values. Numbers in bold indicate *p* values < 0.05.

Var.	Factor	Num *df*	Den *df*	sumsq	meansq	*F*	*p*
lipase	Value_TMinus1	1	23	8.58e+05	8.58e+05	6.44	**0.018**
	Timepoint	2	48	2.44e+07	1.22e+07	91.41	< **0.001**
	Fertilizer	2	23	8.34e+06	4.17e+06	31.32	< **0.001**
	PlasticType	2	23	2.89e+05	1.44e+05	1.08	0.355
	Block	3	23	1.07e+06	3.57e+05	2.68	0.071
	Fertilizer:PlasticType	4	23	2.87e+05	7.18e+04	0.54	0.708
	Timepoint:Fertilizer	4	48	4.34e+06	1.09e+06	8.15	< **0.001**
	Timepoint:PlasticType	4	48	1.18e+05	2.95e+04	0.22	0.925
	Timepoint:Block	6	48	8.5e+05	1.42e+05	1.06	0.397
	Timepoint:Fertilizer:PlasticType	8	48	4.92e+05	6.15e+04	0.46	0.877
	Value_TMinus1	1	23	6.86e+02	6.86e+02	0.12	0.732
	Timepoint	2	48	6.18e+05	3.09e+05	54.38	< **0.001**
	Fertilizer	2	23	3.37e+04	1.69e+04	2.97	0.071

Var.	Factor	Num *df*	Den *df*	sumsq	meansq	*F*	*p*
ß-glucosidase	PlasticType	2	23	1.12e+04	5.59e+03	0.98	0.389
	Block	3	23	2.88e+04	9.61e+03	1.69	0.197
	Fertilizer:PlasticType	4	23	2.91e+04	7.28e+03	1.28	0.307
	Timepoint:Fertilizer	4	48	9.62e+04	2.4e+04	4.23	**0.005**
	Timepoint:PlasticType	4	48	2.8e+03	7.01e+02	0.12	0.973
	Timepoint:Block	6	48	1.95e+04	3.25e+03	0.57	0.75
	Timepoint:Fertilizer:PlasticType	8	48	9.02e+04	1.13e+04	1.98	0.069
ß-xylosidase	Value_TMinus1	1	23	3.66e+02	3.66e+02	2.33	0.141
	Timepoint	2	48	2.53e+04	1.26e+04	80.23	**< 0.001**
	Fertilizer	2	23	1.9e+03	9.52e+02	6.05	**0.008**
	PlasticType	2	23	1.11e+02	5.55e+01	0.35	0.707
	Block	3	23	1.46e+03	4.88e+02	3.10	**0.047**
	Fertilizer:PlasticType	4	23	2.44e+02	6.11e+01	0.39	0.815
	Timepoint:Fertilizer	4	48	2.5e+03	6.24e+02	3.96	**0.007**
	Timepoint:PlasticType	4	48	2.58e+02	6.45e+01	0.41	0.801
	Timepoint:Block	6	48	1.16e+03	1.94e+02	1.23	0.307
	Timepoint:Fertilizer:PlasticType	8	48	1.6e+03	2e+02	1.27	0.282
phenoloxidase	Value_TMinus1	1	23	4.92e+06	4.92e+06	1.54	0.227
	Timepoint	2	48	1.27e+08	6.36e+07	19.88	**< 0.001**
	Fertilizer	2	23	2.27e+07	1.14e+07	3.55	**0.045**
	PlasticType	2	23	1.63e+06	8.14e+05	0.25	0.777
	Block	3	23	4.21e+07	1.4e+07	4.38	**0.014**
	Fertilizer:PlasticType	4	23	1.26e+07	3.14e+06	0.98	0.436
	Timepoint:Fertilizer	4	48	1.61e+07	4.03e+06	1.26	0.299
	Timepoint:PlasticType	4	48	5.05e+06	1.26e+06	0.40	0.811
	Timepoint:Block	6	48	1.87e+07	3.12e+06	0.98	0.452
	Timepoint:Fertilizer:PlasticType	8	48	1.27e+07	1.59e+06	0.50	0.852
C_{mic}	Value_TMinus1	1	23	5.43e+04	5.43e+04	44.60	**< 0.001**
	Timepoint	2	48	2.29e+04	1.14e+04	9.39	**< 0.001**
	Fertilizer	2	23	4.17e+03	2.08e+03	1.71	0.203
	PlasticType	2	23	3.63e+03	1.82e+03	1.49	0.246
	Block	3	23	1.73e+04	5.75e+03	4.72	**0.01**
	Fertilizer:PlasticType	4	23	6.69e+03	1.67e+03	1.37	0.274
	Timepoint:Fertilizer	4	48	1.03e+04	2.58e+03	2.12	0.093
	Timepoint:PlasticType	4	48	8.77e+03	2.19e+03	1.80	0.144
	Timepoint:Block	6	48	2.17e+04	3.61e+03	2.97	**0.015**
	Timepoint:Fertilizer:PlasticType	8	48	5.25e+03	6.56e+02	0.54	0.821
N_{mic}	Value_TMinus1	1	23	4.9e+02	4.9e+02	17.38	**< 0.001**
	Timepoint	2	48	2.54e+03	1.27e+03	45.03	**< 0.001**
	Fertilizer	2	23	8.73e+00	4.36e+00	0.15	0.857
	PlasticType	2	23	2.18e+02	1.09e+02	3.87	0.036
	Block	3	23	8.44e+02	2.81e+02	9.98	**< 0.001**
	Fertilizer:PlasticType	4	23	3.22e+01	8.04e+00	0.29	0.884
	Timepoint:Fertilizer	4	48	4.48e+02	1.12e+02	3.97	**0.007**
	Timepoint:PlasticType	4	48	4.63e+02	1.16e+02	4.10	**0.006**
	Timepoint:Block	6	48	4.17e+02	6.94e+01	2.46	**0.037**
	Timepoint:Fertilizer:PlasticType	8	48	1.66e+02	2.08e+01	0.74	0.658
	Value_TMinus1	1	23	1.57e-04	1.57e-04	0.01	0.922

Var.	Factor	Num *df*	Den *df*	sumsq	meansq	*F*	*p*
N-acetyl-glucosaminidase	Timepoint	2	48	3.46e+00	1.73e+00	107.80	< **0.001**
	Fertilizer	2	23	8.42e-02	4.21e-02	2.63	0.094
	PlasticType	2	23	1.79e-02	8.97e-03	0.56	0.579
	Block	3	23	2.16e-02	7.2e-03	0.45	0.721
	Fertilizer:PlasticType	4	23	2.73e-02	6.83e-03	0.43	0.788
	Timepoint:Fertilizer	4	48	2.79e-01	6.98e-02	4.35	**0.004**
	Timepoint:PlasticType	4	48	1.78e-02	4.45e-03	0.28	0.891
	Timepoint:Block	6	48	2.09e-01	3.48e-02	2.17	0.062
	Timepoint:Fertilizer:PlasticType	8	48	1.2e-01	1.5e-02	0.93	0.498

Table S7-4. ANOVA table for silage maize biomass and grain yield of summer barley with variable (Var.), numerator degrees of freedom (*df*), sum of squares (sumsq), mean sum of squares (meansq), *F* and *p* values. Numbers in bold indicate *p* values < 0.05.

Var.	Factor	*df*	sumsq	meansq	*F*	*p*
Silage maize - biomass	Fertilizer	2	1.92e-01	9.58e-02	0.01	0.987
	PlasticType	2	3.95e+01	1.97e+01	2.69	0.089
	Block	3	5.71e+01	1.9e+01	2.59	0.076
	Fertilizer:PlasticType	4	1.13e+01	2.82e+00	0.38	0.818
	Residuals	24	1.76e+02	7.35e+00		
Summer barley - grain yield	Fertilizer	2	2.71e+00	1.35e+00	4.92	**0.016**
	PlasticType	2	1.17e+00	5.83e-01	2.12	0.142
	Block	3	1.27e+01	4.22e+00	15.37	< **0.001**
	Fertilizer:PlasticType	4	9.58e-02	2.39e-02	0.09	0.986
	Residuals	24	6.6e+00	2.75e-01		

Table S7-5. Simple contrasts of *Fertilizer* or *PlasticType* within *Timepoint* levels. Absolute and relative differences (Diff.) between treatment groups with lower and upper 95 % confidence intervals (CI low, CI up), *t* statistics, *df* degrees of freedom and *p* values. Numbers in bold indicate *p* values < 0.05.

Var.	Contrast	*Timepoint*	Diff.	CI low	CI up	Diff. (%)	CI low (%)	CI up (%)	*t*	*df*	*p*
lipase	Compost - Control	M1	1077.59	718.33	1436.85	58.25	38.83	77.67	7.18	70.89	< **0.001**
	Digestate - Control		147.24	-209.47	503.95	7.96	-11.32	27.24	0.99	70.97	0.587
	Digestate - Compost		-930.35	-1289.69	-571.00	-31.78	-44.05	-19.50	-6.20	70.88	< **0.001**
	Compost - Control	M5	858.76	499.50	1218.02	62.46	36.33	88.59	5.72	70.89	< **0.001**
	Digestate - Control		505.45	148.74	862.16	36.76	10.82	62.71	3.39	70.97	**0.003**
	Digestate - Compost		-353.31	-712.66	6.04	-15.82	-31.91	0.27	-2.35	70.88	0.055
	Compost - Control	M17	145.13	-214.12	504.39	5.10	-7.52	17.71	0.97	70.89	0.6
	Digestate - Control		254.27	-102.44	610.98	8.93	-3.60	21.46	1.71	70.97	0.21
	Digestate - Compost		109.14	-250.21	468.48	3.65	-8.36	15.66	0.73	70.88	0.748
	Compost - Control	M1	2.83	-9.43	15.10	7.48	-24.92	39.87	0.55	70.97	0.845
	Digestate - Control		0.26	-12.01	12.52	0.68	-31.71	33.08	0.05	70.97	0.999

Var.	Contrast	*Timepoint*	Diff.	CI low	CI up	Diff. (%)	CI low (%)	CI up (%)	*t*	*df*	*p*
ß-xylosidase	Digestate - Compost		-2.57	-14.83	9.69	-6.32	-36.45	23.81	-0.50	70.97	0.87
	Compost - Control	M5	11.82	-0.44	24.09	47.32	-1.76	96.40	2.31	70.97	0.061
	Digestate - Control		14.98	2.71	27.24	59.94	10.86	109.02	2.92	70.97	**0.013**
	Digestate - Compost		3.15	-9.11	15.41	8.57	-24.74	41.87	0.62	70.97	0.812
	Compost - Control	M17	-6.48	-18.75	5.78	-9.86	-28.51	8.80	-1.27	70.97	0.42
	Digestate - Control		14.89	2.62	27.15	22.64	3.99	41.30	2.91	70.97	**0.013**
	Digestate - Compost		21.37	9.11	33.63	36.05	15.37	56.74	4.17	70.97	< **0.001**
ß-glucosidase	Compost - Control	M1	9.58	-64.53	83.69	3.95	-26.58	34.48	0.31	70.91	0.949
	Digestate - Control		3.23	-70.51	76.98	1.33	-29.05	31.71	0.10	70.97	0.994
	Digestate - Compost		-6.35	-80.80	68.10	-2.52	-32.02	26.99	-0.20	70.83	0.977
	Compost - Control	M5	80.62	6.51	154.73	46.77	3.78	89.76	2.60	70.91	**0.03**
	Digestate - Control		58.62	-15.13	132.36	34.00	-8.78	76.78	1.90	70.97	0.145
	Digestate - Compost		-22.01	-96.46	52.44	-8.70	-38.12	20.73	-0.71	70.83	0.76
	Compost - Control	M17	-53.90	-128.01	20.21	-13.94	-33.10	5.23	-1.74	70.91	0.197
	Digestate - Control		68.31	-5.43	142.06	17.66	-1.40	36.73	2.22	70.97	0.075
	Digestate - Compost		122.21	47.76	196.66	36.71	14.35	59.08	3.93	70.83	< **0.001**
N_{mic}	LDPE - Control	M1	-0.62	-5.89	4.65	-3.02	-28.70	22.66	-0.28	70.70	0.957
	(PLA/PBAT) - Control		-3.16	-8.48	2.17	-15.37	-41.29	10.55	-1.42	70.42	0.336
	(PLA/PBAT) - LDPE		-2.54	-7.78	2.70	-12.74	-39.04	13.56	-1.16	70.85	0.481
	LDPE - Control	M5	-8.19	-13.47	-2.92	-36.56	-60.08	-13.03	-3.72	70.70	**0.001**
	(PLA/PBAT) - Control		-0.14	-5.46	5.19	-0.61	-24.36	23.13	-0.06	70.42	0.998
	(PLA/PBAT) - LDPE		8.06	2.82	13.30	56.65	19.82	93.48	3.68	70.85	**0.001**
	LDPE - Control	M17	-0.16	-5.44	5.11	-0.57	-18.88	17.74	-0.07	70.70	0.997
	(PLA/PBAT) - Control		2.97	-2.35	8.29	10.31	-8.17	28.79	1.34	70.42	0.38
	(PLA/PBAT) - LDPE		3.13	-2.10	8.37	10.94	-7.35	29.23	1.43	70.85	0.33
N_{mic}	Compost - Control	M1	0.76	-4.54	6.06	3.78	-22.40	29.95	0.35	70.56	0.936
	Digestate - Control		-3.68	-8.92	1.56	-18.16	-44.05	7.72	-1.68	70.84	0.22

Var.	Contrast	*Timepoint*	Diff.	CI low	CI up	Diff. (%)	CI low (%)	CI up (%)	*t*	*df*	*p*
	Digestate - Compost		-4.44	-9.70	0.81	-21.14	-46.14	3.86	-2.02	70.79	0.114
	Compost - Control	M5	4.58	-0.72	9.88	26.39	-4.13	56.91	2.07	70.56	0.103
	Digestate - Control		2.23	-3.01	7.47	12.86	-17.32	43.04	1.02	70.84	0.567
	Digestate - Compost		-2.35	-7.60	2.90	-10.70	-34.64	13.23	-1.07	70.79	0.535
	Compost - Control	M17	-2.12	-7.42	3.18	-7.25	-25.40	10.89	-0.96	70.56	0.606
	Digestate - Control		3.71	-1.54	8.95	12.69	-5.26	30.63	1.69	70.84	0.215
	Digestate - Compost		5.82	0.57	11.08	21.50	2.10	40.89	2.65	70.79	**0.026**

Table S7-6. Contrasts of N-acetyl-glucosaminidase activities by Timepoint. Statistical tests are performed on log-transformed data. The ratios of the contrasts and 95 % confidence intervals (CI low, CI up) are back-transformed to the original scale of the data. *t* statistics, *df* degrees of freedom and *p* values. Numbers in bold indicate *p* values < 0.05.

	Contrast	*Timepoint*	Ratio	CI low	CI up	*t*	df	*p*
N-acetyl-glucosaminidase	Compost / Control	M1	1.03	0.76	1.40	0.25	68	0.967
	Digestate / Control		0.92	0.68	1.24	-0.66	68	0.789
	Digestate / Compost		0.89	0.66	1.21	-0.90	68	0.642
	Compost / Control	M5	1.30	0.96	1.76	2.10	68	0.097
	Digestate / Control		1.59	1.18	2.16	3.71	68	**0.001**
	Digestate / Compost		1.22	0.90	1.66	1.59	68	0.255
	Compost / Control	M17	0.82	0.60	1.10	-1.61	68	0.249
	Digestate / Control		1.14	0.85	1.55	1.07	68	0.539
	Digestate / Compost		1.40	1.04	1.90	2.67	68	**0.026**

Table S7-7. Main effect contrasts of *Fertilizer* for grain yield of summer barley and phenoloxidase activity. Absolute and relative differences (Diff.) between treatment groups with lower and upper 95 % confidence intervals (CI low, CI up. *t* statistics, *df* degrees of freedom and *p* values. Numbers in bold indicate *p* values < 0.05.

Var.	Contrast	Diff.	CI low	CI up	Diff. (%)	CI low (%)	CI up (%)	*t*	*df*	*p*
Grain yield – summer barley	Compost - Control	-0.14	-0.68	0.39	-2.21	-10.49	6.07	-0.67	24	0.785
	Digestate - Control	0.50	-0.04	1.03	7.70	-0.58	15.98	2.32	24	0.072
	Digestate - Compost	0.64	0.11	1.17	10.13	1.67	18.60	2.99	24	**0.017**
Phenol-oxidase	Compost - Control	-25.07	-1283.02	1232.89	-0.35	-17.95	17.25	-0.05	23	0.999
	Digestate - Control	1185.34	-80.72	2451.40	16.58	-1.13	34.30	2.34	23	0.069
	Digestate - Compost	1210.40	-57.11	2477.91	16.99	-0.80	34.79	2.39	23	0.063

Supplementary figures

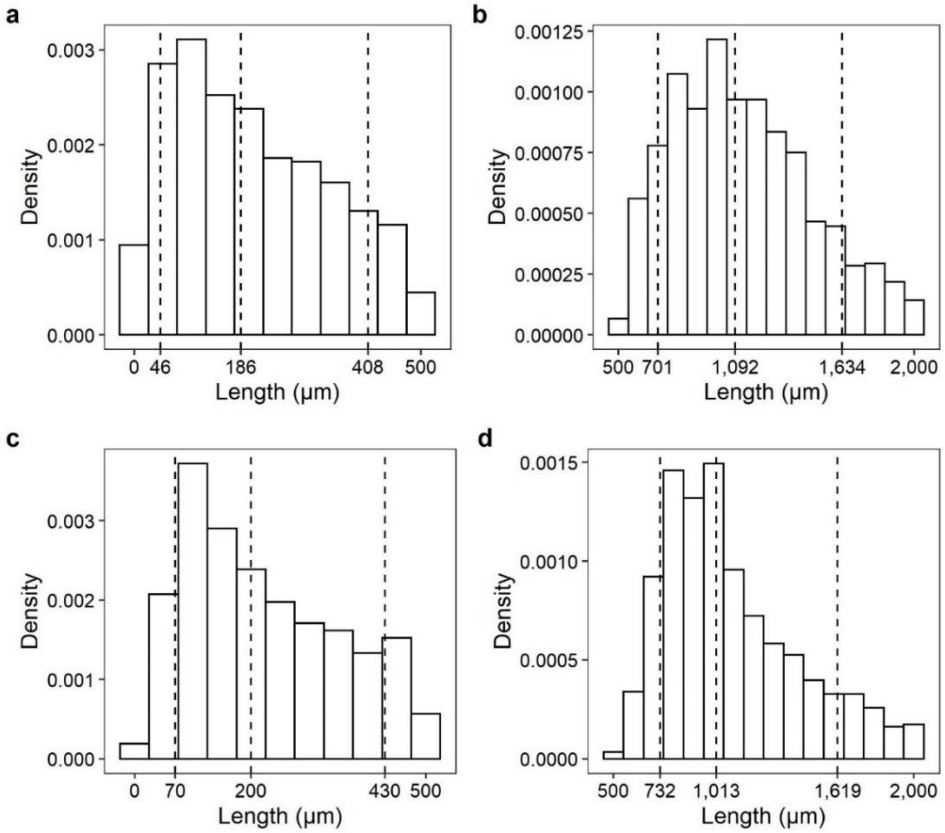

Fig. S7-1. Particle size distributions of MP particles in MP-soil-mixtures of **a** LDPE < 0.5 mm (*n* = 4774), **b** LDPE 0.5 – 2 mm (*n* = 1053), **c** PLA/PBAT < 0.5 mm (*n* = 1274), and **d** PLA/PBAT 0.5 – 2 mm (*n* = 857). The dashed lines show the 10th, 50th (median), and 90th percentiles from left to right.

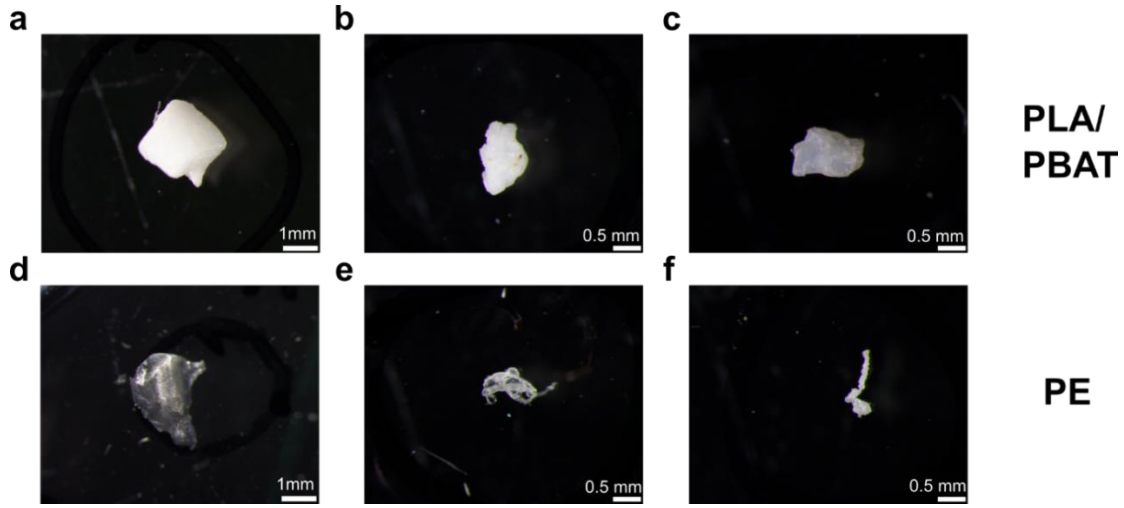

Fig. S7-2. Representative microscopic images of MP > 0.5 mm as initially added to the field detected in the MP-soil-mixtures. **a – c** PLA/PBAT, **d – f** PE.

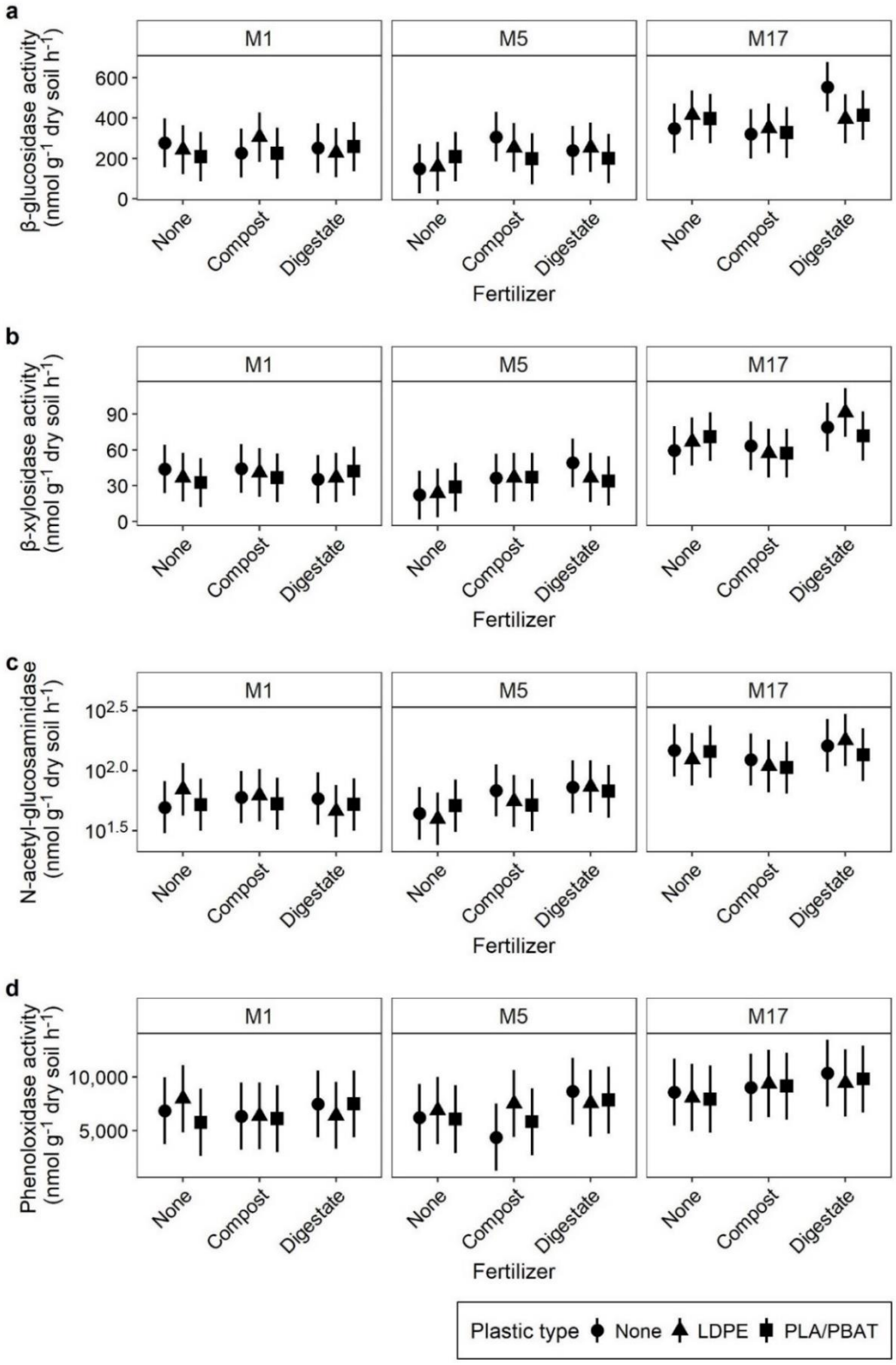

Fig. S7-3. a ß-glucosidase **b** ß-xylosidase **c** N-acetyl-glucosaminidase, and **d** phenoloxidase activity as a function of MP and organic fertilizers one month (M1), five (M5), and 17 months (M17) after the addition of 2 g m^{-2} MP. Data are presented as estimated marginal means (n = 4) with lower and upper 95 % confidence intervals (error bars). Note that data of N-acetyl-glucosaminidase are log-transformed.

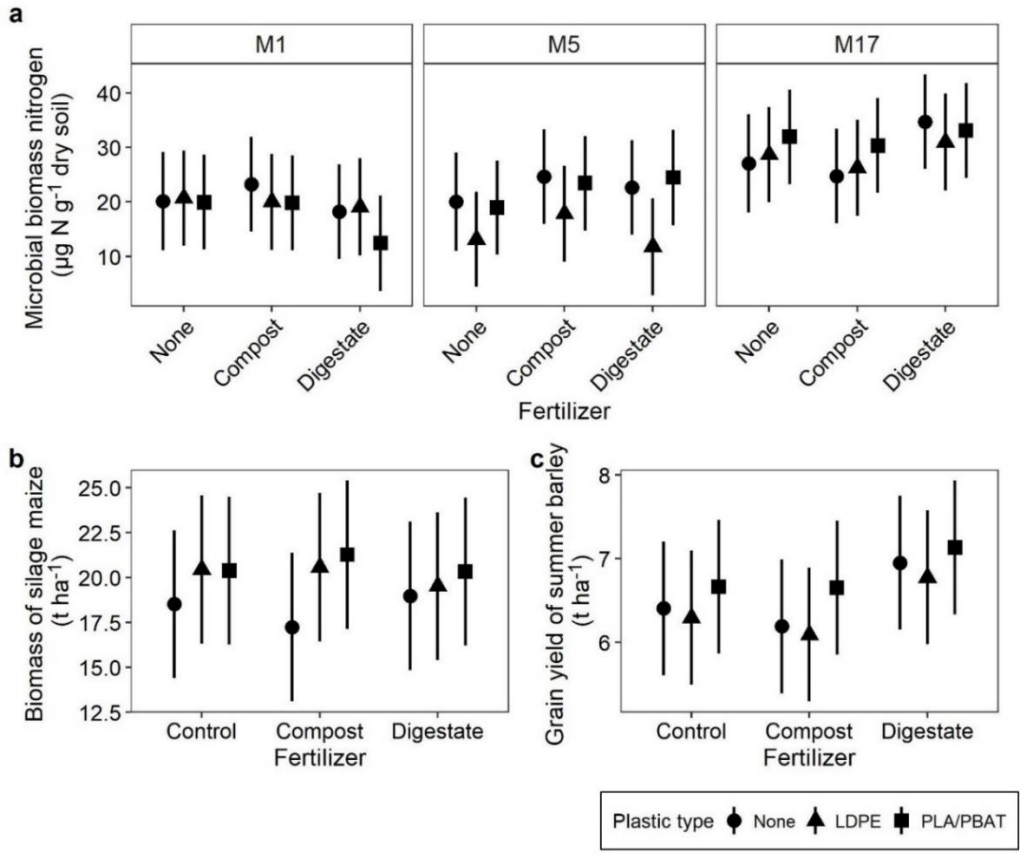

Fig. S7-4. a Microbial biomass nitrogen, **b** biomass of silage maize, and **c** grain yield of summer barley as a function of MP and organic fertilizers after one month (M1), five (M5) and 17 months (M17) after the addition of 2 g m^{-2} MP. Data are presented as estimated marginal means (n = 4) with lower and upper 95 % confidence intervals (error bars).

Fig. S7-5. Land machine harvesting summer barley on the site of the field experiment. Possible entry of red varnish particles due to abrasion of the red protective coating.

8 Microplastics effects on reproduction and body length of the soil-dwelling nematode *Caenorhabditis elegans*

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8.1 Abstract

Microplastics (MP) are pervasive in the environment. There is ample evidence of negative MP effects on biota in aquatic ecosystems, though little is known about MP effects in terrestrial ecosystems. Given numerous entry routes of MP into soils, soil organisms are likely to be exposed to MP. We compared potential toxicological effects of MP from (i) low-density polyethylene (LDPE) (mean diameter ± standard deviation: 57 ± 40 µm) and (ii) a blend of biodegradable polymers polylactide (PLA) and poly(butylene adipate-co-terephthalate) (PBAT) (40 ± 31 µm) on the reproduction and body length of the soil-dwelling bacterivorous nematode *Caenorhabditis elegans*. Feed suspensions without (control) or with MP (treatments) at concentrations of 1, 10, and 100 mg MP L^{-1} were prepared and nematodes were exposed to those suspensions on agar plates until completion of their reproductive phase (~ 6 days). Using Nile red-stained PLA/PBAT MP particles and fluorescence microscopy, we demonstrated the ingestion of MP by *C. elegans* into pharynges and intestines. Under MP exposure, nematodes had fewer offspring (up to 22.9 %) compared to nematodes in the control group. This decline was independent on the plastic type. We detected a tendency toward greater decreases in offspring at higher concentrations. Despite hints of negative effects on nematode body length under MP exposure, we could not derive a consistent pattern. We conclude that in MP-contaminated soils, the reproduction of nematodes, central actors in the soil food web, can be affected, with potentially negative implications for key soil functions, e.g., the regulation of soil biogeochemical cycles.

8.2 Introduction

Microplastics (MP) have only recently been recognized as an environmental threat to terrestrial ecosystems. MP are plastic particles smaller than 5 mm, and with different shapes such as spheres, fibers, and fragments (de Souza Machado et al., 2018; Rillig et al., 2019). Although reliable data on the prevalence of MP in soils is scarce (Watteau et al., 2018), soils are presumably large sinks for MP and MP may harm soil organisms (Bläsing and Amelung, 2018; Hurley and Nizzetto, 2018).

It has been shown for aquatic organisms such as mussels, langoustines, copepods, short crabs, and lugworms that the ingestion of MP can lead to negative effects on growth, reproduction and survival (Galloway et al., 2017; Foley et al., 2018; Franzellitti et al., 2019). These detrimental effects can be nutritional, a result of lower food intake resulting in energy deficiencies (Franzellitti et al., 2019), but also physical, due to lacerations and inflammations (Horton et al.,

2017). In contrast, little is known about MP effects on soil fauna (Chae and An, 2018). Early studies on earthworms under MP exposure indicated that some biological functions could be inhibited (Cao et al., 2017; Huerta Lwanga et al., 2016). One study documented histopathological damage, including congestion fibrosis and inflammatory infiltrates in earthworms in response to MP exposure, although no biological functions were affected (Rodriguez-Seijo et al., 2017). Wang et al. (2019) found oxidative stress in earthworms in response to artificially high MP exposure only. Translocation of MP by earthworms (Huerta Lwanga et al., 2017a; Rillig et al., 2017b) and collembola (Maaß et al., 2017) could increase the exposure of other soil-dwelling species to MP. In addition, evidence on the accumulation of MP from soil to earthworms to chicken feces (Huerta Lwanga et al., 2017b) indicates that MP may enter terrestrial food webs through trophic transfers.

Nematodes (roundworms) live in any terrestrial habitat, exhibit high diversity across soils (Yeates and Bongers, 1999), and have a wide range of diets (Yeates et al., 1993; Orgiazzi et al., 2016). By regulating biogeochemical cycles and ecosystem processes, including mineralization and decomposition of organic matter (Bardgett et al., 1999; Ferris 2010), they are key organisms in the soil food web. The soil-dwelling bacterivorous nematode *Caenorhabditis elegans*, distributed world-wide, is a well-established model organism for ecotoxicological tests of different kinds of pollutants such as mycotoxins, persistent organic pollutants and endocrine-disrupting compounds (Leung et al., 2008; Keller et al., 2018; Chen et al., 2019) and has been used for biosafety assessments of nanoparticles (Wu et al., 2019). Fang-Yen et al. (2009) observed that *C. elegans* can ingest polystyrene beads that can further be transported into the intestine, a possible entry route of MP into the soil food web (Rillig et al., 2017a). An early study on exposure of *C. elegans* to MP reported that MP could lead to inhibition of survival rates, body length, and reproduction, as well as intestinal damage and oxidative stress. While MP effects were independent on plastic type, MP effects were dependent on particle size (Lei et al., 2018b): MP particles of one µm led to the strongest effects when three different sizes (0.1, 1, and 5 µm) were compared.

Biodegradable plastics are considered an environmentally friendly alternative to conventional plastics, as they theoretically can be completely metabolized by microorganisms without leaving plastic residues in the environment (Bandopadhyay et al., 2018; Sander, 2019). For instance, polylactide (PLA) and poly(butylene adipate-co-terephthalate) (PBAT) are common components of biodegradable plastic films which can be substituted for low-density polyethylene (LDPE) films (Künkel et al., 2016). However, there is some evidence that even nominally biodegradable plastics tend to disintegrate instead of being mineralized (de Souza

Machado et al., 2018). A recent study demonstrated that even after 3 years, large quantities of commercially available, biodegradable plastic carrier bags were still present in soils and other environmental compartments (Napper and Thompson, 2019).

Our study aimed to compare possible effects of irregularly shaped MP particles of the conventional plastic type LDPE and a biodegradable blend of PLA/PBAT on nematodes. We evaluated the biological endpoints of reproduction and body length in the model organism *C. elegans* in response to MP exposure at different concentrations. We hypothesized that (i) *C. elegans* can ingest MP and (ii) MP adversely affect reproduction and body length of *C. elegans*, with stronger negative impacts at higher MP concentrations. Furthermore, we expected to observe comparable effects of both conventional and biodegradable MP.

8.3 Materials and methods

Microplastic preparation and characteristics

Two types of plastics were used in the experiment: 1) low-density polyethylene (LDPE) in the form of granules (Lupolen 2420 H, LyondellBasell Industries N.V., Rotterdam, Netherlands) and 2) a blend consisting of the polymers polylactide (PLA, Ingeo™ Biopolymer 7001D, NatureWorks LLC, Minnetonka, Minnesota, United States) and poly(butylene adipate-co-terephthalate) (PBAT, Ecoflex® F Blend C1200, BASF SE, Ludwigshafen, Germany) with a mixing ratio of 80/20 % w/w compounded at the "Institut für Kunststofftechnik" (University of Stuttgart, Stuttgart, Germany).

We used irregularly shaped MP particles because a non-spherical shape can be expected due to input of fragmented MP from mulch film and plastic bag residues in compost into soils. The particle size ingestible by *C. elegans* is smaller than 3 µm (Fang-Yen et al., 2009). However, it is very challenging, to produce irregularly shaped MP particles with defined size distribution < 50 µm. For our experiments, plastic granules were first ground to MP particles < 5 mm with a speed rotor mill (Pulverisette, Fritsch GmbH, Idar-Oberstein, Germany) and later milled to smaller fragments using a cryomill with liquid nitrogen (Cryomill, Retsch, Haan, Germany) at the Fraunhofer Institute for Chemical Technology (Pfinztal, Germany). This procedure yielded particle sizes of 57 ± 40 µm (LDPE) and 40 ± 31 µm (PLA/PBAT) (Fig. 8-1). Due to light microscopy detection limits at ≤ 3 µm (see "Supplementary Material 1"), we could not differentiate particles ≤ 3 µm. The proportions of particles ≤ 3 µm (ingestible for *C. elegans*) were 8.0 % (LDPE) and 7.4 % (PLA/PBAT). Particles of both plastic types were similarly shaped, as shown by their form factors (Fig. S8-1). A detailed description of particle characteristics is given in "Supplementary Material 1".

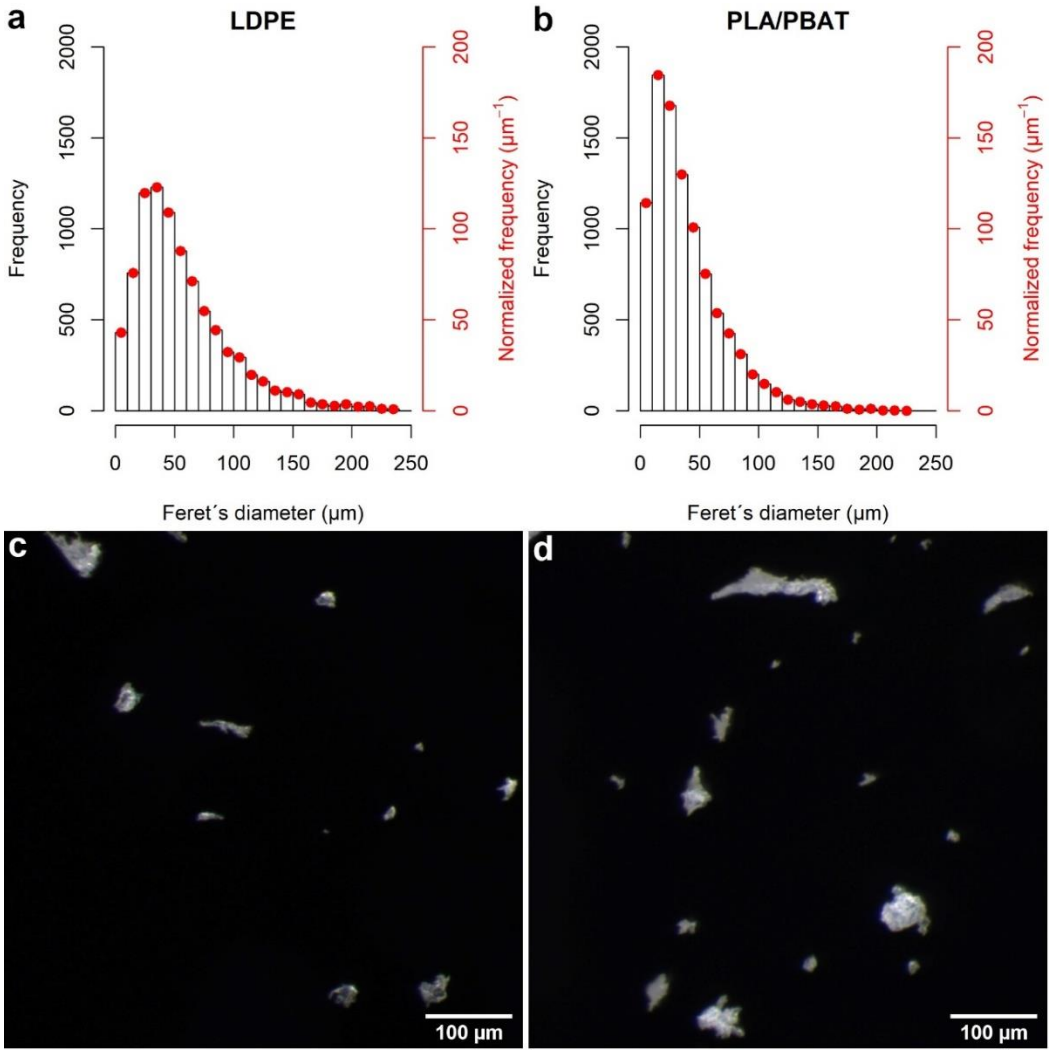

Fig. 8-1. The particle size distribution of **a** LDPE and **b** PLA/PBAT. The second ordinate shows the normalized frequency according to Filella (2015). Micrograph of **c** LDPE and **b** PLA/PBAT.

Cultivation of C. elegans and preparation of MP feed suspensions

We used the *C. elegans* wild-type strain N2 in our assays, which was obtained from the Caenorhabditis Genetics Center (University of Minnesota). *C. elegans* was fed with *Escherichia coli* OP50 and cultivated on Nematode Growth Medium agar plates. For both assays, L1 nematodes were used. L1 refers to nematodes from the first of four larval stages in the life cycle of *C. elegans* before they become fertile adults (Gonzalez-Moragas et al., 2015). For the treatments, MP feed suspensions were prepared at concentrations of 1, 10, and 100 mg MP L^{-1}. The MP feed suspensions consisted of M9 buffer, a common worm buffer for handling *C. elegans* (He, 2011), freshly harvested *E. coli* OP50 from overnight cultures and MP. For the control group, feed suspensions of M9 buffer and freshly harvested *E. coli* OP50

without MP were prepared. All feed suspensions (with and without MP) were then shaken for 30 min and placed in an ultrasonic bath for 15 min to prevent agglomeration of the particles in the treatments. Before an aliquot of 100 µl of the feed suspensions were added to the agar plates, the suspensions were vortexed for 10 s. Following the addition of the feed suspensions to the agar plates, they were dried under the laboratory hood and incubated at 19.5°C for 12 h. The entire procedure was performed under sterile conditions.

Ingestion assay

Nematodes were exposed to Nile red (NR) stained PLA/PBAT particles at a concentration of 100 mg L^{-1}. For this purpose, PLA/PBAT particles were colored with the fluorescent dye NR (72485, Sigma-Aldrich, St. Louis, Missouri, United States) that was recently used for the detection of MP in environmental samples (e.g., Shim et al., 2016; Maes et al., 2017). For staining of the MP particles, a NR stock solution of 1 mg L^{-1} methanol was first prepared. MP particles were stained with a NR working solution of 10 µg mL^{-1} by shaking the suspensions at 35 rpm for 1 h on a laboratory shaker (Roto-Shake Genie®, Scientific Industries, Inc., New York, United States). After incubation, the suspensions were transferred to glass Petri dishes where the solvents evaporated under the laboratory hood. The stained MP particles were used for the preparation of MP feed suspensions. Five nematodes were taken from a pre-culture and added to Nematode Growth Medium agar plates prepared with MP feed suspensions. The adult nematodes were transferred to new plates after 3 and 6 days, respectively. After 9 days of incubation, images were taken with a fluorescence microscope using an excitation wavelength of 510 – 560 nm and emission wavelength > 590 nm (Axiophot with filter set 487914 / analogous to current filter set 14, Carl Zeiss Microscopy GmbH, Jena, Germany). The Feret's diameter of MP particles incorporated by *C. elegans* was determined using Fiji 1.52p (Schneider et al., 2012; Schindelin et al., 2012). For better visualization, the particles were pseudo-colored on the images.

Reproduction and body length assay

To exclude potential side effects of NR, here we only used non-stained MP particles. The experimental design consisted of two plastic types (LDPE or PLA/PBAT) at three different concentrations (1, 10, and 100 mg L^{-1}) and a control without MP addition (each $n = 8$).

L1 nematodes were individually picked from a pre-culture with a smoothed platinum picker and placed on the agar plates (one nematode per plate) prepared with feed suspensions with MP (treatments) or without (control). Subsequently, the nematodes were exposed to these feed

suspensions on the agar plates at 19.5 ° C until oviposition (~ 3 days). At intervals of 24 hours the nematodes were transferred to new agar plates prepared with the nutrient suspensions with MP (treatments) or without (control) until the end of the reproduction phase (~ 3 days). In total, nematodes of the treatment groups were exposed to MP for 6 days.

Nematode offspring per 24 h were counted optically with a stereomicroscope (Nikon SMZ1000, Nikon, Tokyo, Japan). The body length of the adult nematodes that survived the reproduction phase was determined using a stereomicroscope with camera (Zeiss Axio Scope.A1 & AxioCam ICc 5, Carl Zeiss Microscopy GmbH, Jena, Germany) and Fiji 1.52p. Before taking images, the nematodes were anesthetized with 20 mM tetramisole hydrochloride (L9756, Sigma-Aldrich, St. Louis, Missouri, United States).

Statistics

For data analysis, we used the statistical software R (R Core Team, 2018). To examine our hypotheses, we fitted a one-way analysis of variance model to our data and specified linear contrasts of interest as proposed by Mangiafico (2015) using the "glht" command from the R package "multcomp" (Hothorn et al., 2008). To test for an effect of MP on reproduction and body length, we compared each treatment to the control group. To clarify differences between the plastic types, we compared LDPE and PLA/PBAT per concentration level. To test the assumption that higher concentrations would lead to stronger effects, we compared the higher to the lower concentrations for each plastic type. We adjusted the *p* values according to Benjamini and Hochberg (1995), to correct for several comparisons. Following the recommendation of Wasserstein et al. 2019, we did not define a significance level and deliberately omitted the term "statistically significant". The results of the statistical tests are given in Table S8-2. We confirmed the model assumptions of the ANOVA visually by residual diagnostics plots (Kozak and Piepho, 2018).

We only considered nematodes that remained alive until the completion of the reproductive phase. Underdeveloped worms and worms that died before completing the reproductive phase from unexplained causes of death (e.g., mechanical damage) were excluded from the analysis. This resulted in an unbalanced design with at least $n = 5$ (Table S8-1).

8.4 Results

Ingestion Assay

We confirmed the uptake of NR stained PLA/PBAT MP particles in the pharynx and posterior intestinal lumen of *C. elegans* by fluorescence microscopy (Fig. 8-2a&b). The particles ingested by *C. elegans* displayed in Fig. 8-2, in the pharynx and intestine had a Feret's diameter of 2.3 – 5.1 µm and 1.3 – 2.5 µm, respectively.

Surprisingly, internal hatching of larvae was observed in a nematode with particles in the pharynx (Fig. 8-2c). This phenomenon could also be observed in four nematodes from the reproduction assay exposed to 10 (n = 1) and 100 mg L^{-1} LDPE (n = 2) and 100 mg L^{-1} PLA/PBAT (n = 1). These individuals were not considered in the statistical analysis because they died before the completion of the reproductive phase. In the control group without MP, internal hatching did not occur.

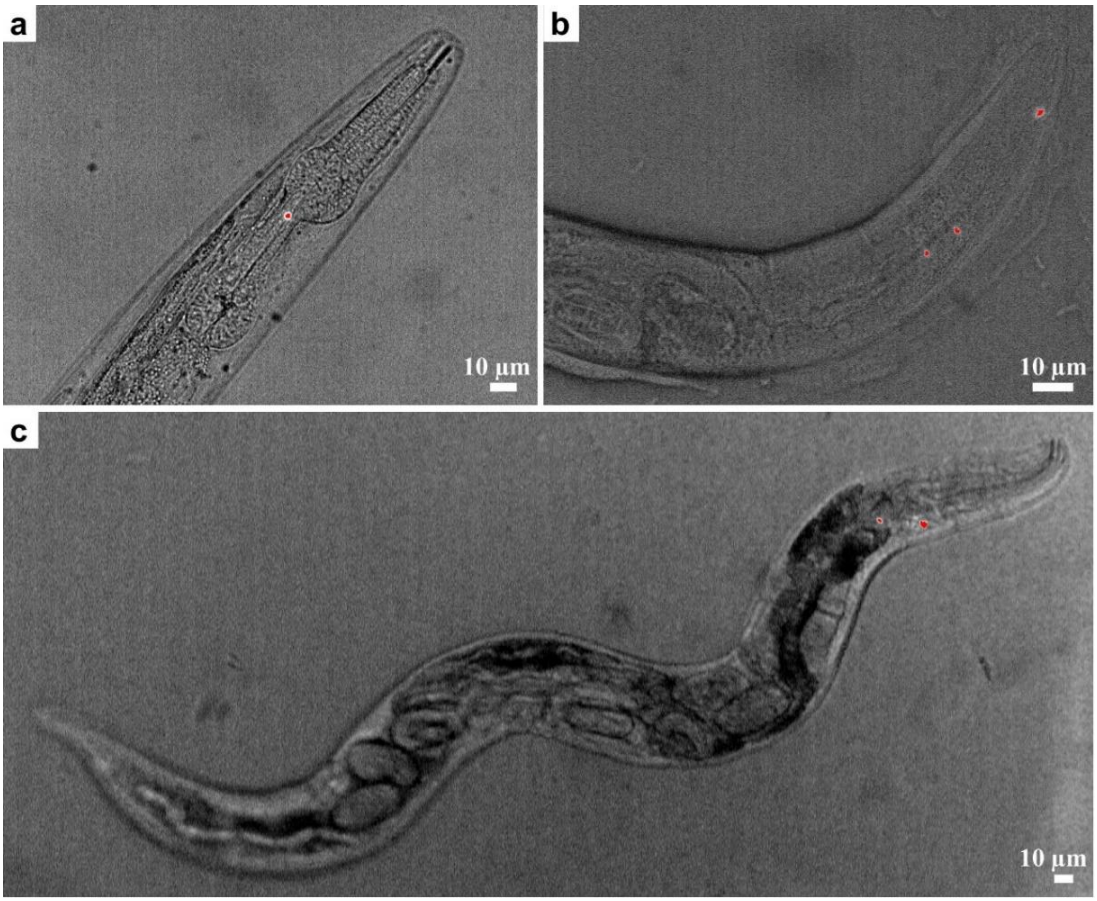

Fig. 8-2. Ingestion of NR-stained PLA/PBAT particles (red) in the **a** pharynx and **b** intestine lumen of *C. elegans*. **c** Nematode with NR-stained PLA/PBAT particles in the pharynx and internal hatching.

Reproduction and body length assay

Number of nematode offspring in the control group was 267 ± 6 (mean ± standard error) (Fig. 8-3a). Under MP exposure, nematodes produced 4.6 – 22.9% fewer offspring than nematodes in the control group. The strongest reduction in comparison to the control group was found at 10 mg L^{-1} LDPE (p = 0.03). For both plastic types, we observed a tendency towards stronger declines at higher concentrations. Under exposure to 10 and 100 mg L^{-1} compared to 1 mg L^{-1} LDPE, offspring declined by 18.4% (p = 0.08) and 9.9% (p = 0.37) stronger relative to the control. Exposure to 10 and 100 mg L^{-1} compared to 1 mg L^{-1} PLA/PBAT, resulted in declines relative to the control which were by 4.7% (p = 0.63) and 6.5% (p = 0.49) stronger. We found only marginal differences in offspring between LDPE and PLA/PBAT at all concentration levels (Table S8-2). The coefficient of variation (CV) of the treatment groups (9.2 – 17.5%) was higher than the CV of the control group (5.5%).

In the control group, body length of the nematodes was 1470 ± 24 µm (Fig. 8-3b). We could not observe a clear pattern for the body length of nematodes exposed to MP. Body length decreased most strongly relative to the control at exposure level of 1 mg L^{-1} PLA/PBAT (14.5%, $p = 0.06$) and at both 1 mg L^{-1} (7.8%, $p = 0.25$), and 10 mg L^{-1} (8.8%, $p = 0.21$) LDPE. Observed body lengths in all other treatments were close to the body length of the control group. Body length was not influenced by plastic type. With a CV of 4.1%, the control group exhibited lesser variance than the treatment groups, the CVs of which ranged from 4.7 to 14.2%.

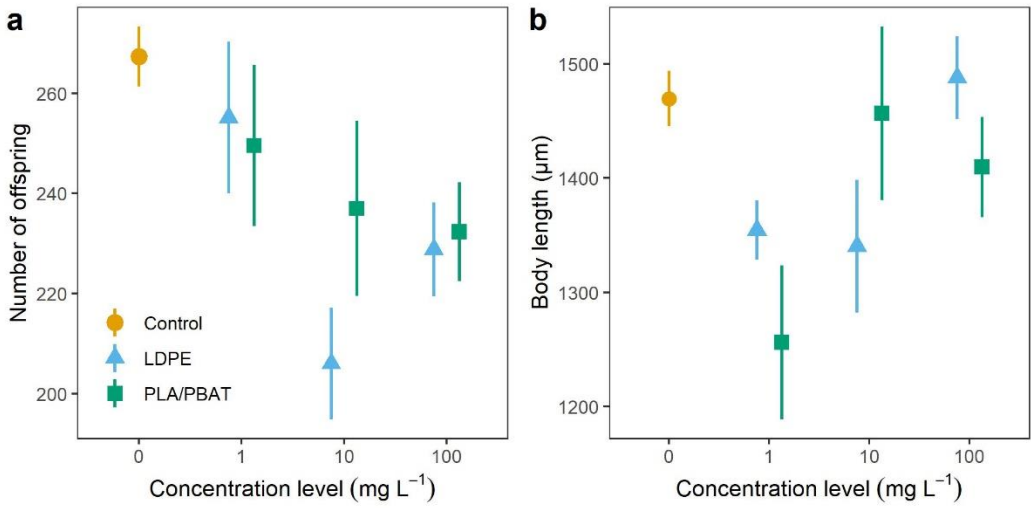

Fig. 8-3. a Number of offspring and **b** body length of *C. elegans* as a function of concentration and plastic type compared to the control group. Data are presented as means ± SE.

8.5 Discussion

By demonstrating that *C. elegans* can and does ingest MP particles, we established one prerequisite for the potential development of toxic effects (Horton et al., 2017; Kim and An, 2019) in *C. elegans*. Particle ingestion is generally controlled by a species-specific particle-to-mouth size ratio that defines the size of the particles which are ingestible by a particular species (Horton et al., 2017). Recently, Mueller et al. (2020) provided evidence for earlier findings from Fang-Yen et al. (2009) that the ingestible particle size of *C. elegans* is limited by the size of the buccal cavity, which in their study developed to 4.4 ± 0.5 µm. While polystyrene (PS) beads < 3 µm entered the entire intestinal system of *C. elegans*, particles > 6 µm did not enter the body of the nematodes at all (Mueller et al., 2020). In agreement with these findings, we detected in our study MP particles with sizes of 2.3 – 5.1 µm and 1.3 – 2.5 µm in the pharynges and intestines of *C. elegans*, respectively.

We found that MP reduced offspring of *C. elegans* by 4.5 – 22.9%, with a tendency towards greater declines in offspring at higher MP concentrations. The strong decline in offspring at

10 mg L^{-1} compared to the control might indicate the existence of a critical effect concentration of MP. Contra-intuitively, the decrease was more pronounced at 10 mg L^{-1} LDPE than at 100 mg L^{-1} LDPE which we attribute to the statistical uncertainties of the comparison between these treatments (p = 0.44, see Table S8-2). The existence of a critical effect concentration is supported by observations in Lei et al. (2018a), who found clear lethal effects of PS beads in *C. elegans* at concentrations higher than 5 mg L^{-1}. Mueller et al. (2020) established clear dose-response curves for the reproduction of nematodes exposed to PS beads from 0.1 to 10 µm, with smaller particles exerting a stronger toxicity. Only 0.1 µm-sized particles caused effects on nematode reproduction with an EC_{50} at 77 mg L^{-1} (all units converted) below the highest concentration level of 100 mg L^{-1} used in our study, while larger particles caused inhibitions in reproduction at considerably higher concentrations (Mueller et al., 2020). Lei et al. (2018b) compared the toxic effects of five different MP types of irregular shape (mean diameter: ~ 70 µm) on body length, embryo number, brood size, calcium levels in the intestine, and the expression of stress-indicating enzyme activity of *C. elegans*. Additionally, they compared the ingestion and toxic effects of PS beads of different sizes (0.1, 1, and 5 µm). Lei et al. (2018b) found offspring reductions of 2.4 – 28.0 %, which were similar to the offspring reductions observed in our study. They found that body length was reduced by 4.9 – 11.4 % when nematodes were exposed to MP, while we could not observe a clear MP effect pattern, as some MP treatments showed reductions compared to the control, while others did not.

Given the estimated fraction of MP particles < 3 µm used in our experiments of 8.0 (LDPE) and 7.4% (PLA/PBAT), the concentration levels of 1, 10 and 100 mg L^{-1} in our study translate into 0.08, 0.8, 8.0 mg L^{-1} and 0.074, 0.74, to 7.4 mg L^{-1} in the ingestible range for *C. elegans.* Thus, in Mueller et al. (2020) the likelihood of an ingestion of MP by *C. elegans* was much higher than in our study, since they used spherical particles in the size range of 0.1 – 3 µm at concentrations of 40 - 12,500 mg L^{-1}. The authors found effects on reproduction at considerably higher concentrations than > 100 mg L^{-1} (exception: 0.1 µm-sized particles with an EC_{50} of 77 mg L^{-1}), which indicates that the observed toxicity of MP in our study at much lower concentrations of ingestible particles was not solely attributed to the ingestion of particles. While direct toxicity of ingested MP particles was shown for 1 µm particles that were preferably ingested and accumulated in the intestines of *C. elegans* (Lei et al., 2018b), there is some experimental evidence that negative effects on reproduction of *C. elegans* are regulated by the ratio of the total surface area of MP particles to the volume of the medium, where the MP is contained (Mueller et al., 2020). Yet, the underlying mechanism of such a surface-related toxicity still needs to be clarified. Mueller et al. (2020) proposed as possible mechanisms of the

surface-related toxicity the binding of food bacteria to MP and a dilution of food bacteria by MP, which could result in a limitation of food availability for the nematodes. In our study, surface-related toxicity could explain, why the MP particles reduced offspring although most of the particles were not directly ingestible by nematodes. Due to their higher specific surface area, irregularly shaped particles (used in our study) presumably lead to higher surface-related toxicity at lower concentrations compared to spherical MP particles (used in Mueller et al., 2020). Hence, toxic effects of MP are also likely to be controlled by particle shape.

In line with Lei et al. (2018b), negative effects on the reproduction of *C. elegans* under MP exposure occurred irrespective of the plastic type. Since LDPE and PLA/PBAT particles in our study had a similar shape and size, the observed reductions in offspring were probably attributable to physical effects, such as intestinal damage (Lei et al., 2018b) or indirect nutritional effects due to interaction of particles with the food bacteria (Mueller et al., 2020). Natural particles such as mesoporous SiO_2 particles, which can be found in soils, can also be ingested by *C. elegans* (Acosta et al., 2018). However, only exposure to nano-sized particles led to reductions in lifespan, mobility, and reproduction, while micro-sized particles showed no effect (Acosta et al., 2018). Mueller et al. (2020) compared the effects of SiO_2 particles to PS beads of equal size and showed that SiO_2 particles exhibited a clearly lower toxicity, which was indicated by toxic effects at significantly higher particle-to-bacteria ratio. The authors claimed that the specific density of a material played an important role for the toxicity, because particles with specific density in the range of the bacteria like plastics would be taken up more readily.

We found a greater variance within our MP treatments than in the control treatment for both offspring and body length. One possible explanation is that not all nematodes exposed to MP had ingested them, such that only worms that ingested MP were affected. This was not possible to check, however, as we used non-stained MP in the reproduction and body length assay, and this was not detectable under the microscope inside the nematode bodies. Furthermore, it is possible that the total surface of the particles in the MP feed suspensions differed between the replications within and among the groups due to discrepancies in particle compositions present in the respective suspensions.

Studies of MP effects on other soil-dwelling animals have also reported negative effects of MP on some biological functions, with other functions unaffected. For instance, under high MP exposure, mortality level of the earthworm *Lumbricus terrestris* increased, growth inhibited, and biomass reduced, whereas even at higher concentrations no effect on reproduction was found (Huerta Lwanga et al., 2016). In comparison, Rodriguez-Seijo et al. (2017) observed no adverse effects on survival, reproduction, or body weight of the earthworm *Eisenia andrei*, but

did find gut damage and histological alterations including congestion and fibrosis. Under MP exposure, the earthworm *Eisenia fetida* showed only marginal effects, with the anti-oxidase system negatively affected only at artificially high concentrations (Wang et al., 2019), while the isopod *Porcellio scaber* was not affected by MP at all (Jemec Kokalj et al., 2018). In view of our results and those from these studies, it appears that not all species or their biological endpoints are sensitive to MP, as our findings indicate that body length is a non-sensitive metric for MP toxicity in *C. elegans*. The fact that some biological endpoints were affected by MP and others were not may be explained by the disposable soma theory (Kirkwood, 1977). According to this theory, an organism can try to compensate for stress, in our case induced by MP. This stress and its compensation may lead to an imbalance of biological functions, as one function decreases at the expense of maintenance of another.

Remarkably, in the ingestion assay, we observed that several nematodes that ingested MP (not quantified, however) exhibited internal hatching (Fig. 8-2c). In the reproduction assay, four nematodes which had been exposed to MP and died during the exposure also showed internal hatching. Generally, the phenomenon of internal hatching, also referred to as matricide, can occur under conditions of stress, e.g., starvation, exposure to toxins, or presence of bacteria (Mosser et al., 2011; Pestov et al., 2011). Nematodes, supposedly in response to stress, lay their eggs internally to ensure survival of their offspring, as the offspring receive sufficient nutrients in the body of the adult nematode. However, in our study, it was not clear whether internal hatching was induced directly by MP. Kiyama et al. (2012) observed that in the presence of food, uptake of MP was strongly reduced. Conversely, under the condition of food deficiency, more particles were taken up. A possible scenario in our study could have been that a combined effect of MP particle consumption and starvation would have led to internal hatching. The potential link of MP uptake and internal hatching should be investigated quantitatively in future studies.

We are aware that in our approach exposure of *C. elegans* to MP was rather artificial (MP feed suspensions on agar plates). We chose this exposure, though, because we aimed at understanding the general potential of MP to develop a toxicity in *C. elegans*. In soils, it would not have been possible to achieve a general process understanding. The design of more realistic experimental setups with soils is challenging because currently there are only few data on MP contamination in soils and it is not clear whether the MP concentrations found to date in urban (0.3 – 67.5 g kg^{-1}), riparian (0 – 0.055 g kg^{-1}), and agricultural soils (0 – 42,960 particles kg^{-1}) (Helmberger et al., 2019) lead to negative effects on soil biota. Due to detection limits, even less data is available in environmental samples for MP particles < 10 µm (Haegerbaeumer et

al., 2019) and thus for the ingestible size range of *C. elegans*. Given the aliquot of 100 µl that we gave to each nematode in our experiments, one nematode was effectively exposed to 0.1, 1 and 10 µg of MP. Based on a global median abundance of 12,030 individuals per 1 kg dry soil (van den Hoogen et al., 2019) and the mass-based MP concentrations reported in soil (see above), this translates into an exposure to 0 – 4.6 µg per nematode for riparian and 25 – 5,600 µg per nematode for soils in industrial areas. Thus, the applied MP amounts in our study are in a range typically found in natural soils.

8.6 Conclusion

We found that nematodes can ingest MP particles which might negatively affect their reproduction. Toxic effects of MP on nematode reproduction in soils cannot be ruled out. The toxicity risk for conventional and biodegradable MP particles is likely to be the same, as MP toxicity is rather attributable to physical and indirect nutritional effects rather than to chemical effects. Although we have hints of negative effects of MP on the body length of nematodes, our results are not conclusive. Since nematodes, as key members of the soil food web, may be at risk under MP exposure, our results suggest potentially negative implications for important soil functions, e.g., the regulation of biogeochemical cycles. Further studies are needed to estimate critical effect concentrations and to elucidate the influence of particle shape for nematodes under realistic exposure scenarios in soils.

8.7 Data availability statement

The datasets generated for this study are available on request to the corresponding author.

8.8 Author contributions

LS, SM, EK, RM, HP, and LR contributed conception and design of the study. LS performed the ingestion assay as well as the reprodution and body length assay supervised by RM and LR. LS conducted the data evaluation and statistics supported by HP. US determined the size distribution and characteristics of the microplastic particles supervised by FB. LS wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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8.12 Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2020.00041/full#supplementary-material

Particle size distribution and shape characteristics

Particle size distributions (Fig. S8-1) and shape characteristics of MP particles were obtained by static image analysis (International Organization for Standardization 2014) using a stereomicroscope with a camera (Leica MZ16 FA & Leica DFC420 C, Leica Microsystems, Heerbrugg, Switzerland). Images were processed with a customized macro in image analysis software Fiji 1.52p (Schindelin et al. 2012; Schneider et al. 2012) including a thresholding procedure based on Brocher (2014). Particles were then measured with the morphology plugin by Landini (2008). According to Shekunov et al. (2007), data entries with a Feret's diameter ≤ 3 µm were completely removed due to the measurement range of light microscopes using R software (R Core Team 2018). In addition, particles > 99.7 % percentile were excluded as they showed heavy agglomeration. In this way, a total of 8.51 % (LDPE) and 7.47 % (PLA/PBAT) of data entries were filtered out. Values of circularity and elongation were removed when they exceeded the maximum values of the theoretical model as proposed by Kröner and Doménech Carbó (2013). Finally, shape characteristics (elongation, circularity, and convexity) were calculated as described in Crompton (2005).

Particles of LDPE and PLA/PBAT were characterized as irregularly shaped based on a circularity of 0.53 ± 0.15 and 0.57 ± 0.16, elongation of 0.36 ± 0.16, 0.34 ± 0.16, and convexity of 0.84 ± 0.09 and 0.86 ± 0.09, respectively (Fig. S8-1).

Supplementary figures

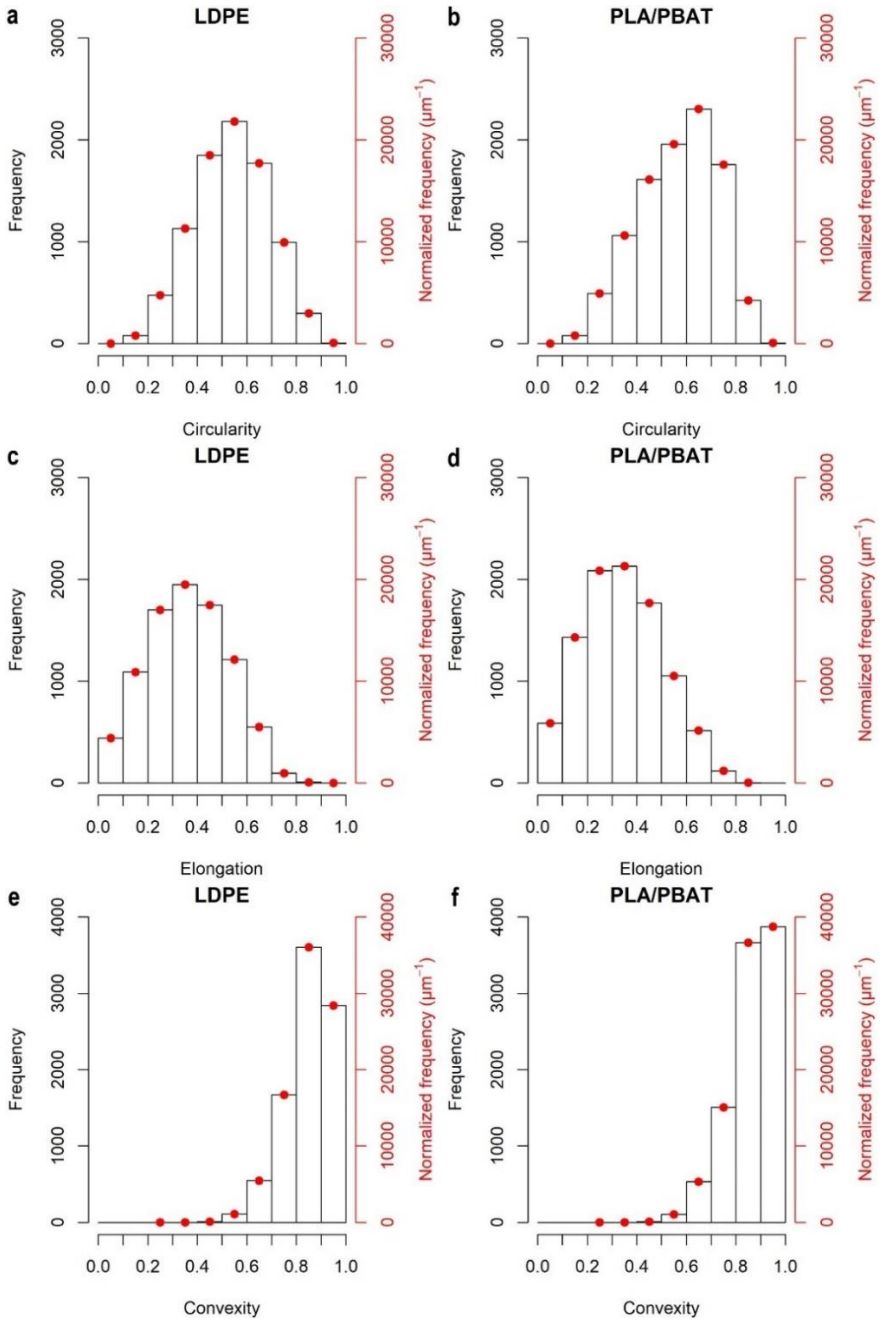

Fig. S8-1. Shape characteristics of LDPE (*n* = 8790) and PLA/PBAT (*n* = 9694) particles. Circularity, elongation, and convexity of LDPE (**a**, **c**, **e**) and PLA/PBAT (**b**, **d**, **f**) respectively. Three entries per plastic type were removed by the Kröner and Doménech Carbó (2013) algorithm. Circularity measures how much a particle deviates from a perfect circle. Values closer to one indicate a spherical shape, while values closer to zero are typical for fibers and irregularly shaped particles. Elongation measures the aspect ratio ration of particles. Values closer to one indicate a very small width-to-length ration, while values closer to zero are typical for particles with a circular or rectangular surface. Note that particles with both a rough and a smooth surface might have equal elongations. Convexity measures the surface roughness of particles. Values close to one will be found in case of smooth surfaces, while values closer to zero are typical for irregularly shaped particles. Note that one cannot differentiate between fibers and spheres when both have the same convexity.

Supplementary tables

Table S8-1. Experimental design of reproduction and body length assay with number of replicates (*n*) used in the statistical analysis, means, standard errors (SE), and coefficients of variation (CV).

Plastic type	Concentration level (mg L^{-1})	Offspring				Body length			
		n	Mean	SE	CV (%)	*n*	Mean (µm)	SE (µm)	CV (%)
Control	0	6	267	6.0	5.5	6	1470	24.3	4.1
LDPE	1	6	255	15.1	14.5	6	1354	25.9	4.7
	10	6	206	11.1	13.2	6	1340	58.0	10.6
	100	5	229	9.4	9.2	5	1488	36.0	5.4
PLA/PBAT	1	7	250	16.1	17.0	7	1256	67.2	14.2
	10	5	237	17.5	16.5	5	1457	75.9	11.6
	100	6	232	9.9	10.5	5	1410	44.0	7.0

Table S8-2. Specified comparisons of means between the treatments of reproduction and body length assay with difference and [lower; higher] 95% confidence interval including *p* value. LDPE: low-density-polyethylene. PLA/PBAT: blend of biodegradable polymers polylactide (PLA) and poly(butylene adipate-co-terephthalate) (PBAT). Indices denote the concentration levels of 0, 1, 10 and 100 mg L^{-1}.

	Offspring		**Body length**	
Comparison between treatments	**Difference**	***p* value**	**Difference (µm)**	***p* value**
$LDPE_1$ vs. $Control_0$	-12 [-67; +43]	0.63	-115 [-333; +103]	0.25
$LDPE_{10}$ vs. $Control_0$	-61 [-116; -6]	0.03	-129 [-347; +89]	0.21
$LDPE_{100}$ vs. $Control_0$	-39 [-96; +19]	0.23	+18 [-210; +247]	0.86
$PLA/PBAT_1$ vs. $Control_0$	-18 [-71; +35]	0.49	-213 [-423; -3]	0.06
$PLA/PBAT_{10}$ vs. $Control_0$	-30 [-88; +27]	0.30	-13 [-242; +216]	0.86
$PLA/PBAT_{100}$ vs. $Control_0$	-35 [-90; +20]	0.23	-60 [-289; +169]	0.59
$LDPE_{10}$ vs. $LDPE_1$	-49 [-104; +6]	0.08	-14 [-232; +204]	0.86
$LDPE_{100}$ vs. $LDPE_{10}$	+23 [-35; +80]	0.44	+147 [-81; +376]	0.21
$LDPE_{100}$ vs. $LDPE_1$	-26 [-84; +31]	0.37	+133 [-95; +362]	0.21
$PLA/PBAT_{10}$ vs. $PLA/PBAT_1$	-13 [-68; +43]	0.63	+200 [-21; +422]	0.07
$PLA/PBAT_{100}$ vs. $PLA/PBAT_{10}$	-5 [-62; +53]	0.85	-47 [-286; +192]	0.69
$PLA/PBAT_{100}$ vs. $PLA/PBAT_1$	-17 [-70; +36]	0.49	+153 [-68; +375]	0.21
$LDPE_1$ vs. $PLA/PBAT_1$	+6 [-47; +59]	0.85	+98 [-112; +308]	0.27
$LDPE_{10}$ vs. $PLA/PBAT_{10}$	-31 [-89; +27]	0.30	-116 [-345; +112]	0.25
$LDPE_{100}$ vs. $PLA/PBAT_{100}$	-4 [-61; +54]	0.85	+78 [-161; +317]	0.49

9 General discussion

9.1 The persistence of microplastics in soil implies long-term exposure of soil organisms to microplastics.

The evaluation of the risk of soil organisms being exposed to MP was based on investigations of the background concentrations of MP, the potential biodegradation of conventional and biodegradable MP (LDPE and PLA/PBAT) in a microcosm approach, and concentration changes of these MP in the field after addition to an agricultural soil.

MP concentration data were only available for agricultural soils that faced MP inputs associated with their specific management history, including the application of organic fertilizers, sewage sludges, or plastic mulches (*Joos* and *Tender*, 2022). The exception was the study by *Piehl* et al. (2018) that showed that the topsoil (0 – 5 cm) of a conventionally managed agricultural field, without the specific management history mentioned above, contained 0.34 MP particles kg^{-1}. However, in their study it remained unclear how frequently MP particles < 1 mm occurred in the soil, as these were not analyzed. Evidence from my field experiment provides relevant data on MP concentrations in soil with conventional agricultural management without using MP-containing fertilizers, sewage sludges, and plastic mulches in the past. The field experiment's agricultural topsoil (0 – 10 cm) contained 9 different MP types with ~ 300 particles kg^{-1} in the fraction of MP < 0.5 mm. Consistent with the study by *Piehl* et al. (2018), the most frequent MPs were PE, PP, PS, and particles – economically significant polymers (*PlasticsEurope*, 2021). Remarkable was the presence of red varnish particles > 0.5 mm in the soil of the field experiment. The varnish particles likely originated from coatings of agricultural machinery that may have become brittle due to weathering and peeled off over the years (e.g., exposure to UV light) (*Krueger* et al., 2015). This indicates a possible newly discovered input pathway of MP into agricultural soils through the abrasion of machinery coating during tillage. While this has yet to be confirmed for other agricultural soils, it demonstrates the importance of analyzing MP background levels and that not all entry pathways of MP into agricultural soils have yet been identified.

Since significant MP input pathways (MP-containing fertilizers, sewage sludges, and plastic mulches) at the site can be ruled out, the occurrence of MP suggests that MP entered the soil from diffuse sources such as atmospheric deposition, which has been reported for cities, and river catchments, as well as remote areas (*Allen* et al., 2019; *Kernchen* et al., 2022; *Dris* et al., 2015; *Dris* et al., 2016). In addition, in-situ generation of MP through abrasion of machine

coatings during tillage and field operations, as well as resulting from the fragmentation of larger plastic residues from littering due to weathering, could contribute to the input of MP to agricultural soils. Soil organisms face MP exposure levels of 1,200 particles kg^{-1} in agricultural soils treated with sewage sludges and plastic mulches (*Büks* and *Kaupenjohann*, 2020); in soils treated with compost soil organisms are exposed to ~ 900 particles kg^{-1} (*van Schothorst* et al., 2021). The results of my field experiment show that soil organisms can also be exposed to significant concentrations (~ 300 particles kg^{-1}) of various MP even in soils without these specific management practices. Soil monitoring campaigns will help provide robust data on the status quo of MP contamination in agricultural soils and identify spatial distribution patterns associated with specific land use practices and anthropogenic activities.

If the input rates of MP exceed the transformation rates of MP, MP persist in the soil (*Krueger* et al., 2015). Soil organisms would be permanently exposed to MP. This typically applies to most conventional plastics, such as PP, PS, and PE, as demonstrates their omnipresence in soils (*Zhang* et al., 2021). These polymers exhibit several immanent constraints to biodegradation such as high molecular weight and non-hydrolyzability (*Krueger* et al., 2015; *Restrepo-Flórez* et al., 2014). Therefore, it is not surprising that LDPE-MP persisted in the soil under laboratory and field conditions. In contrast, biodegradable MP can be transformed into CO_2, H_2O, and microbial biomass by microorganisms; this is biodegraded (*Agarwal*, 2020). However, in the microcosm study, the hydrolyzable PLA/PBAT-MP were biodegraded only slowly (up to 15% MP-C mineralization within 230 days at 25 °C) and only in dry soil (pF 4), where microorganisms found better growth conditions compared to wet soil (pF 2). In laboratory soil incubation experiments, films of PLA/PBAT blends also showed slow biodegradation (~18 % C mineralization for a blend with 45/55 % within 126 days, *Freitas* et al., 2017; ~10 % C mineralization for a blend with 75/25 % w/w within 180 days, *Palsikowski* et al., 2018). The similar range of C mineralization of these PLA/PBAT films (thickness of 0.3 – 1 mm) and the observation in my study that PLA/PBAT particles of the larger size fraction (0.5 – 2 mm) were slightly more mineralized than particles from the smaller size fraction (< 0.5 mm) contradict the assumption that MP biodegradability is enhanced with lower size (i.e., higher specific surface area) in soil.

Biodegradation of commercially available biodegradable mulch films with PBAT as the main component and proportions of PLA or starch proceeds similarly slow (12 – 20 % C mineralization after a 1-year soil incubation; *Anunciado* et al., 2021a). PHAs, including PHB and PHBV are considered the biodegradable polymers with the highest biodegradability in soils and other environments (*Meereboer* et al., 2020; *Resch* et al., 2020). The microcosm study

showed that PHB was almost completely mineralized within 230 days. Bacteria synthesize PHAs under growth limitations serving as storage compounds and thus occur naturally in soils (*Mason-Jones* et al., 2019). However, high brittleness and poor flexibility of PHB and PHBV restrict their application (*Meereboer* et al., 2020; *Resch* et al., 2020). Blending can improve the properties of PHB and PHBV, making them suitable for several applications, e.g., plastic mulches and organic waste bags, but this is at the expense of biodegradability. In the study of *Anunciado* et al. (2021a), the C mineralization of a PLA/PHA blend was 16 %. In another study, PLA/PHBV blend (70/30 %) showed 32 % after 200 days a slightly higher C mineralization (*Muniyasamy* et al., 2016).

In sum, my findings and those from literature establish that PLA/PBAT-MP and films of similar biodegradable blends in the soil are biodegradable in soil. However, in most cases, biodegradation rates are slow and typically decrease after certain mineralization levels are reached, rarely exceeding 30 % (*Muniyasamy* et al., 2016; *Anunciado* et al., 2021a; *Saadi* et al., 2013; *Palsikowski* et al., 2018). Under field conditions, soil temperature and moisture fluctuate seasonally, and optimal conditions for polymer biodegradation rarely occur; microbial activity may be limited under extreme conditions (e.g., drought and low temperatures). Recent evidence suggests that UV light could accelerate the biodegradation of plastic fragments located at the soil surface due to UV-light-induced polymeric changes in their physicochemical properties (*Anunciado* et al., 2021b; *Anunciado* et al., 2021a). This significantly limits the transferability of laboratory-derived findings and emphasizes the importance of field investigations. Results from the field study highlight that the same number of PLA/PBAT-MP particles were found in the soil after 17 months in the field as were initially added to the soil, with neither significant shifts in size distributions nor visual alterations of the PLA/PBAT particles. This strongly indicates that PLA/PBAT-MP were not significantly biodegraded and persisted in the arable soil. Other studies evaluated the biodegradation of PLA- and PBAT-based films in soils under field conditions by monitoring proxies such as reduction in surface area by visual assessment (i.e., surface area reduction), weight loss, and changes in physicochemical properties because it is not possible to quantify the biodegradation of polymers in soil by respirometric methods under field conditions (*Rudnik* and *Briassoulis*, 2011; *Liao* and *Chen*, 2021; *Sintim* et al., 2020). PLA films persisted in Mediterranean soil for 11 months with slight traces of disintegration and fragmentation (*Rudnik* and *Briassoulis*, 2011). Within three years, commercially available biodegradable mulch films based on PLA and PBAT showed a surface-area reduction between 26 to 83 %, while FTIR indicated enzymatic hydrolysis of the ester bonds (*Sintim* et al., 2020). Weight loss of neat PLA, PBAT, and PBAT/PLA (90/10 % w/w) after six months in a field soil

was 8, 7, and 2 % (*Liao* and *Chen*, 2021). While my results suggest no biodegradation of PLA/PBAT-MP, these studies show that at least some biodegradation of PLA- and PBAT-based polymers can occur under field conditions. However, given the methods to assess degradation such as visual surface area reduction, the question arises whether the polymers were actually degraded or whether these only fragmented and formed MP as a result of biodegradation (*Helmberger* et al., 2020; *Agarwal*, 2020). As long as biodegradable MPs are only slowly and not completely biodegraded under field conditions, the issue of MP formation is not resolved. Due to slowly proceeding biodegradation, surface areas could increase (*Helmberger* et al., 2020), with a higher potential for interactions with soil organisms, such as uptake by soil fauna, but also for sorption of chemicals, including other classes of pollutants, with consequences not yet foreseeable (*Souza Machado* et al., 2018).

The primary constraint to efficient biodegradation of the PLA/PBAT in my study could be the relatively high PLA proportion (85 %). In soil, neat PLA typically shows low biodegradation (*Palsikowski* et al., 2018; *Muniyasamy* et al., 2016). PLA proved far better biodegradable in compost with up to 90 % mineralization after 200 days (*Tabasi* and *Ajji*, 2015; *Muniyasamy* et al., 2016). The process temperatures of composting (55 – 60 °C) are close to the glass transition temperature of PLA (58 – 60 °C), which then changes to a rubbery state, enabling improved enzymatic hydrolysis by thermophilic microbial consortia (*Meereboer* et al., 2020; *Muniyasamy* et al., 2016). Also, the PLA- and PBAT- based biodegradable mulch films show increased biodegradability when composted (*Anunciado* et al., 2021a; *Muniyasamy* et al., 2016). Recent evidence suggests that PBAT- and PLA-MP remain after industrial composting and fermentation, indicating incomplete biodegradation (*Steiner* et al., 2022). In my studies, I used pristine MP. Still, plastic fragments are physically and biologically altered during composting and fermentation which changes the polymer structure and can promote the accessibility and hydrolysis of enzymes (*Ren* et al., 2019; *Steiner* et al., 2022; *Muniyasamy* et al., 2016; *Meereboer* et al., 2020). With regard to their environmental safety, it is vital to investigate the biodegradability of PBAT and PLA residues from composts and digestates in soil. On the one hand, these could have a higher biodegradability in the soil, analogous to biodegradable mulch films, which had a better degradability in the soil due to physicochemical changes due to weathering (*Anunciado* et al., 2021a). On the other hand, higher crystallinity of the MP fragments could reduce their biodegradability in soil (*Steiner* et al., 2022).

9.2 Microplastics form a specific habitat in the soil.

Soil microbiological indicators (microbial biomass C, PLFAs, and soil enzyme activities) did not suggest interactions with soil microorganisms in the MP-amended soil under laboratory or field conditions. However, zooming into the microscale level, i.e., onto the MP interface, revealed that PLA/PBAT-MP particles exhibited cracks after 230 days of soil incubation. Other studies have documented surface erosion of biodegradable polymers, which is commonly associated with the initial step of biodegradation, i.e., the depolymerization of the polymers by hydrolytic enzymes and abiotic factors, such as soil water (*Sander*, 2019; *Li* et al., 2022; *Lamparelli* et al., 2021). It is well known from the literature that lipases, esterases, and cutinases can catalyze the depolymerization of biodegradable polyesters (*Marten* et al., 2005; *Tokiwa* and *Calabia*, 2007; *Zumstein* et al., 2017). However, the activities of enzymes that can catalyze the depolymerization of these polymers have not yet been quantified on their surface. Studying lipase and ß-glucosidase activities of individual MP – an innovative approach of my thesis – enabled valuable insights into microbial-driven processes in the MP soil interface. Lipase activities on the PLA/PBAT surfaces incubated in soil were significantly higher than on LDPE particles. The highest lipase activities coincided with the highest mineralization degree (~15%) observed for PLA/PBAT incubated in dry soil. These findings suggest that lipases induced the depolymerization of PLA/PBAT particles by enzymatic hydrolysis, which led to the surface erosion of these particles.

Comparing the surface-specific enzyme activities of lipase and ß-glucosidase on MP particles to the bulk soil suggests that PLA/PBAT particles were hotspots for these microbial processes, i.e., the cleavage of the ester bonds of PLA/PBAT and the decomposition of cellobioses to glucose. Among others, lipase-producing Gram-positive bacteria, e.g., Saccharibacteria, known to produce lipases and identified as a member of the PLA-associated microbiome (*Rüthi* et al., 2020), could have released the lipases from adjacent bulk soil. Other studies have established that MP forms ecological niches for certain microorganisms with enrichment of specific taxa on the plastics surfaces or lower biodiversity of the plastics-associated microbiome (*Huang* et al., 2019; *Yi* et al., 2021; *Rüthi* et al., 2020; *Bandopadhyay* et al., 2020; *MacLean* et al., 2021). The study of microbial processes in this anthropogenic habitat (the plastisphere) has been neglected. Results from my thesis identified MP as hotspots for specific microbial processes and support the existence of the plastisphere as novel anthropogenic microbial habitat by process-based evidence. In this specific habitat, microorganisms interacted with MP via their enzymes and paved the way for the utilization of MP (PLA/PBAT). To better understand the ecological role of the plastisphere in soil, other microbial processes in this anthropogenic habitat

should be studied. For instance, in the case of PLA/PBAT, the approach to measuring lipase activities on the MP surfaces could be extended to esterases and cutinases, which have been reported to play a role in the depolymerization of PLA/PBAT (*Marten* et al., 2005; *Zumstein* et al., 2017). Furthermore, metagenomic analyses could be used to investigate how trade-offs between different microbial taxa regulate the community-level metabolic efficiency of MP degradation in the plastisphere. To elucidate the spatial dimensions of the plastisphere, setups with finer resolutions, also considering the proximate bulk soil close to MP particles, could be sampled. This would contribute to a better understanding of the scale-dependency of these processes.

On the larger scale, in PLA/PBAT-amended soil, however, no increased lipase activities were observed compared to MP-free soil. This suggests that the activities of lipases associated with the biodegradation of PLA/PBAT were masked by the naturally occurring decomposition of native lipids as part of the soil organic matter. Most likely due to the slow biodegradation of PLA/PBAT in my study, the influence of the plastisphere on soil microorganisms was spatially restricted. In comparison, PHB that was used as a positive control in the mineralization test showed that the influence of the plastisphere can lead to shifts in the abundance and composition of the main microbial groups (an increase of 19 % of Gram-negative bacteria). Consistent with this finding, PHBV at concentrations of 1 – 20 % in soil induced increases in microbial activity, growth, and enzyme activities, as well as shifts in soil bacterial community associated with metabolic changes (*Zhou* et al., 2021). The authors proposed that these PHBV-related alterations could lead to the enhanced decomposition of native soil organic matter through cometabolism, i.e., the degradation of soil organic matter while utilizing PHBV as an energy source, in other words to a “positive priming” effect (*Rillig* et al., 2021). PHAs (PHB and PHBV) show higher biodegradability in soil compared to PLA/PBAT, as proves the almost complete mineralization in my studies and high biodegradation rates reported in literature (*Meereboer* et al., 2020).

Nevertheless, recent findings from *Meng* et al. (2021) indicate the stimulation of native soil organic decomposition due to PBAT/PLA-MP (85 % PBAT, 10 % PLA, and 5 % calcium) at concentrations of 2 – 2.5 %, evidenced by decreases in soil organic matter, but increases in dissolved organic matter. Presumably, the blend used in *Meng* et al. (2021) showed higher biodegradation in soil compared to the blend used in my thesis, which could be attributed to the higher proportion of PBAT, which is better biodegradable in soil than PLA (*Palsikowski* et al., 2018; *Muniyasamy* et al., 2016; *Saadi* et al., 2013). It is likely that biodegradable MP increase C and nutrient turnover as a function of their biodegradability. If future studies confirm a

sustained acceleration of C turnover and increased CO_2 release due to enhanced microbial activity in the plastisphere, the continuous introduction of biodegradable MP into the soil could amplify climate change effects.

As MP provide surfaces, this implies interactions with microorganisms but also with the soil matrix consisting of aggregates, native soil particles, cations, and anions within the soil solution. The assumptions that MP acted as microhydrological niches (higher lipase activity and mineralization of PLA/PBAT in dry soil) and the observation that brown particles were attached to the PLA/PBAT surface (most likely clay minerals or oxides) suggest that the plastisphere could interfere with the hydrosphere and the mineralosphere (*Kandeler* et al., 2019; *Boeddinghaus* et al., 2021) in soil. Indeed, in addition to soil biological effects, MP have been reported to induce changes in soil physicochemical properties, such as bulk density, porosity, aggregation, electrical conductivity, water holding capacity, pH, and nutrient availability, which in turn can affect microbial communities and soil processes (*Kim* et al., 2020b; *Zhang* et al., 2021). MP-induced physicochemical changes were not in the scope of my thesis, but it cannot be excluded that these occurred in my studies. Nevertheless, since soil microorganisms and microbial functions were not compromised by the presence of MP in soil, the MP concentrations in my studies were presumably below the critical loads at which such effects occur. Recently a study found no effects of LDPE at concentrations of up to 10,000 kg ha^{-1} on microbial abundance and composition, wheat yield and growth, or earthworm abundance and biomass during one growing season (*Brown* et al., 2022). If even such high loads (500 times higher than in the field experiment of my thesis) do not lead to detrimental effects, at least in the short term, this raises the question of whether critical MP loads will realistically be reached all day. For agricultural soils with a similar MP pollution legacy as in my field experiment, it is unlikely that detrimental effects on soil functions occur. However, under other management practices (application of sewage sludges, organic fertilizers, and plastic mulches) and with the accumulation of MP related to high persistence, critical loads cannot be completely ruled out. Also, the importance of physicochemical effects indirectly affecting the soil habitat could increase.

9.3 Microplastics can enter the soil food web by uptake through nematodes.

The nematode study demonstrated the uptake of irregularly shaped biodegradable MP particles through the nematode *C. elegans* in their pharynges and intestines (1.3 – 5.1 µm), which had already been reported for conventional MP fragments and beads (*Fueser* et al., 2019; *Fang-Yen*

et al., 2009; *Mueller* et al., 2020b; *Lei* et al., 2018b). Furthermore, MP caused a reprotoxicity (reduction in offspring up to 23 %), which did not significantly differ between the conventional LDPE and the biodegradable PLA/PBAT. Although there were hints of harmful effects of MP on nematode body length, no consistent toxicity pattern was evident. These findings confirm previous studies that MP can develop general toxicity in nematodes (*Mueller* et al., 2020b; *Lei* et al., 2018b; *Lei* et al., 2018a). In addition to reproduction and body length, in these studies, other biological endpoints have been found to be affected by MP, including survival rates, intestinal damage, oxidative stress, and locomotion behavior.

On the one hand, MP toxicity (irregularly shaped conventional MP) has been attributed to direct physical effects (*Lei* et al., 2018b). This was determined by the intestinal toxicity (based on low calcium levels and high expression levels of intestine stress-related enzymes), which was most pronounced for the most ingested particles (1 µm in size). On the other hand, also indirect nutritional effects have been proposed (*Mueller* et al., 2020b). Nutritional effects would be due to surface-related toxicity caused by interference with food availability, e.g., through binding of food bacteria to MP surfaces. Given these proposed toxicity mechanisms, which have to be confirmed by further studies, the risk for biodegradable MP to develop toxicity (including the endpoints mentioned above) in nematodes appears to be equal to conventional MP. Since most of the MP exposed to nematodes were in a non-ingestible size range (< 5 µm) in my study, indirect nutritional effects are likely to have played a larger role in toxicity. As irregularly shaped MP particles, i.e., higher specific surface area compared to beads, were used here, it is likely that interference with bacteria could have been more intense compared to *Mueller* et al. (2020b).

From a toxicological point of view, an important observation of my field study was the proportion of small MP particles (< 0.2 mm) found in the background of the arable soil. The MP size distribution was skewed to the left, i.e., the frequency of MP particles increased with decreasing size. Given the current detection limit of 10 µm (*Haegerbaeumer* et al., 2019; *Möller* et al., 2020), this suggests that MP in the ingestible size range of MP could be even more frequent in soil. If future advances in MP detection methods, which allow the detection of MP < 10 µm down to the nanometer scale, do indeed find that small MP and nanoplastics are frequent in soil, this would increase the risk for ingestion of MP by nematodes and associated toxic effects (strongest toxicity with one µm-sized MP; *Lei* et al., 2018b).

In my approach, the well-established ecotoxicological model species *Caenorhabditis elegans* was exposed to irregularly shaped LDPE- and PLA/PBAT- MP within feed suspensions on agar plates, thus in a somewhat artificial setup. This was primarily to allow a general understanding

of MP toxicity mechanisms such as MP ingestion by nematodes to be examined, but also for comparability reasons, as most MP studies involving nematodes used similar exposure scenarios (*Lei* et al., 2018a; *Lei* et al., 2018b; *Mueller* et al., 2020a). These results cannot be transferred to exposure to soils. Still evidence suggests that *C. elegans* when exposed to 0.042 and 0.53 µm PS-MP beads in soil, exhibits a more sensitive response, i.e., a more significant reduction in offspring than exposure to liquid media (*Kim* et al., 2020a). Examination of MP exposure of nematodes in five different soils revealed that in addition to MP concentration, soil properties such as texture, bulk density, and cation exchange capacity determine MP toxicity to nematode offspring in the soil. In addition, studying the effects of MP on only one nematode species (*C. elegans*) is, of course, a substantial simplification of the reality of the soil food web with a large number of different nematodes in the soil with different feeding habits occupying different positions and functions (*Joos* and *Tender*, 2022; *Yeates* et al., 1993; *Bardgett* et al., 1999). Recently *Fueser* et al. (2019) established that uptake of MP through nematodes is controlled by feeding type and the size of their buccal cavity (MP were ingested when the buccal cavity to MP size ratio exceeded 1.3). Therefore, no general statements can be made about the effect of MP on nematodes, and the risk at a given MP load for a given size distribution is species-specific. This is corroborated by a recent study that found that the population growth of *C. elegans* under MP exposure was significantly reduced. In contrast, the population growth of two other bacteria-feeding nematode species, *Acrobeloides nanus* and *Plectus acuminatus*, were unaffected (*Mueller* et al., 2020a). Suppose further studies with more realistic exposure scenarios (real soil environments and different nematode species) confirm the uptake of MP by nematodes and the negative effects of MP on nematodes. In that case, MP will likely enter the soil food web posing risks for bioaccumulation and trophic transfer of MP.

9.4 Microplastics in soil – a threat to soil organisms and their functions?

Findings from my thesis underline the ubiquity of MP in agricultural soils. Even agricultural soils without relevant input pathways linked to the management history of the soils, such as the application of MP-containing organic fertilizers, sewage sludges, and plastic mulches, exhibit significant background concentrations of various MP. This suggests that MP originate from diffuse sources, including atmospheric deposition and in-situ fragmentation of larger plastic fragments from littering. In addition, the abrasion of machinery coating during tillage and field operations, as evidenced by the presence of red varnish particles in the soil of my field study, could contribute to MP input into agricultural soil. My findings highlight that soil organisms

are also exposed to several MP in conventionally managed agricultural soils that were not treated with sewage sludges, organic fertilizers, and plastic mulches in the past.

Studying the potential biodegradation of PLA/PBAT-MP revealed that they are biodegraded at slow rates (15 % after 230 days). This is consistent with the low biodegradation of commercial PLA- and PBAT-based mulch films in the literature. In my field study, PLA/PBAT-MP persisted for 17 months in the soil without evidence of significant biodegradation. From these findings, one can conclude that not only conventional (LDPE) but also biodegradable MP with similar chemical composition to that of PLA/PBAT will remain in the soil in the long term (at least for several years). With proceeding biodegradation of biodegradable MP at slow rates, associated with physicochemical alterations and disintegrations of MP, biodegradable MP could pose a higher risk, e.g., of being taken up by soil fauna and of sorption of chemicals.

Based on soil microbiological indicators (microbial biomass C, PLFAs, and soil enzymes), neither the soil microbial abundance, the composition of the main soil microbial groups, nor key microbial processes that drive the organic matter decomposition (C turnover) were affected by MP under both laboratory and field conditions. At the microscale, there was evidence for microbial processes occurring on the MP-soil-interface of particles after 230 days in soil. PLA/PBAT triggered specific microbial processes (lipase activity) at the microscale, i.e., the MP soil interface. The lipase activities on the PLA/PBAT surface most likely contributed to its surface erosion and depolymerization, paving the way for biodegradation. While other studies have established that MP induce microbial habitats in soil by selecting a specific microbiome (plastics-associated microbiome), findings from my thesis support the formation of a microbial habitat in soil – the plastisphere – based on specific microbial processes that were enhanced compared to the bulk soil. It is currently unpredictable which soil ecological and functional implications such microscale changes in the proximity of MP might have in the long term. For example, as most MP are C-rich but contain N and nutrients (*Sander*, 2019), this could lead to a local depletion of N in the plastisphere and changes in native soil microbial processes. In addition, biodegradable MP could stimulate the decomposition of native soil organic matter, thus reinforcing climate change effects due to faster C turnover in soils.

Ecotoxicological tests with the nematode *Caenorhabditis elegans* showed that these also ingest biodegradable MP. Under MP exposure, the reproduction of nematodes was reduced (up to 23 %), with no differences between biodegradable and conventional MP. Given the possible entry of MP via nematode uptake into the soil food web, this could have ecological consequences, as MP could be transferred from one trophic level to another with potential risks for members on higher trophic levels (e.g., earthworms). Also, potentially negative implications

for essential soil functions, e.g., the regulation of biogeochemical cycles, are likely. Studies to clarify the effects of soil food web entry, such as trophic transfer, are essential to uncover potential ecological impacts on the soil food web and its functionality. There remains much uncertainty regarding concentrations of small MP < 0.01 mm and nanoparticles, and methods for their detection in soil are needed to assess the risk adequately for specific nematode communities for uptake and subsequent negative implications for the biological functions of nematodes.

The persistence of conventional but also biodegradable MP is concerning. Limited biodegradation and persistence of MP will lead to accumulation of these particles in the soil, which implies an increased risk for long-term MP exposure levels associated with a higher potential to affect soil organisms and processes. Based on my findings, using biodegradable polymers as an alternative to replacing conventional polymers appears not necessarily beneficial. It should be ensured that biodegradable MP is rapidly biodegraded in soil and leave no residues behind, which bear the risk of negative long-term implications for soil organisms and functions such as C and nutrient cycling.

Currently, MP concentrations in agricultural soil may not exceed critical levels in soil. But one should keep in mind that higher concentrations of MP in agricultural soils are realistic future scenarios; e.g., *Geyer* et al. (2017) estimated that 12,000 Mt of plastics will have been accumulated in the environment by 2050. Thus, exposure levels are likely to rise with continuous inputs of MP into the environment due to specific agricultural practices such as applying plastic mulches, sewage sludges, and organic fertilizers. Additionally, more MP will enter soils via the atmosphere, and other entry pathways are yet to be identified (such as abrasion of machinery coating). Accordingly, the exposure potential of MP to soil organisms will increase, and so will the potential for interactions. Critical thresholds of MP loads to impair soil functions and organisms may not yet be exceeded but could be reached in the future.

MP is not the only hazard to soil functions. Agricultural soils already face a multitude of disturbances, e.g., contamination with chemical pollutants and climate change (*Schaeffer* et al., 2016). Accordingly, the risk of MP impairing soil functions should not be regarded as an isolated problem but in concert with other environmental stressors. For example, the role of MP as a transport vector for chemical pollutants in soil organisms, which can lead to bioaccumulation of these chemicals, is poorly understood in soils (*Zhang* and *Xu*, 2022). In addition, “probably the most important ecological experiment of all time” (Chapter 10, Paul Eldor, Morris, p. 297, *Morris* and *Blackwood*, 2015), global warming could be indirectly influenced by MP pollution in permafrost soils due to light absorbance of MP particles causing

accelerated thawing (*Bergmann* et al., 2022). Even if MP might not have immediate consequences on their own, these examples illustrate that interactions of MP with other stressors have to be considered when assessing the global risk of MP in soil. Following the precautionary principle, MP emissions into the environment should be limited as much as possible to exclude such potential risks of MP interactions with other environmental stressors.

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