Hydrolyzable microplastics in soil—low biodegradation but formation of a specific microbial habitat?

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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 co-adipate terephthalate) blend, PLA/PBAT) and non-hydrolyzable (low-density polyethylene, LDPE) polymers, and the effects of these 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.

Keywords Polymer fragments · Biodegradation in soil · Enzymatic hydrolysis · Soil microorganisms · Aliphatic–aromatic co-polysters · Low-density polyethylene

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 glucosidase 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; Zhang et al. 2019a; 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; Teerapattornchai et al. 2003; Tokiwa and Calabia 2007; Zumstein et al. 2017). Examples of polyesters are the aliphatic poly(lactic acid) (PLA) and polyhydroxybutyrate (PHB), and the aromatic poly(butylene co-adipate 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) usually 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 individual MP particles of MP 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).
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 non-hydrolyzable MP. Polyhydroxybutyrate (PHB) was 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 fraction of 0.5–2 mm, while there was a small difference in the fraction of < 0.5 mm between plastic types (Table S1; Fig. S1). 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 S1; Fig. S2; Fig. S3; Cole 2016; Hartmann et al. 2019). All PHB particles were irregularly shaped (Table S1).

Soil sampling and characteristics

The soil was randomly sampled (0–20 cm, Ap horizon) from an agricultural field of the research station Heidelberg (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 CaCl₂) 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; Yükselen 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 (121.170.1608, AppliedChem 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., Win-ooeki, 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 (Yükselen and Kaya 2006) at a mass ratio of MB added to the soil of 0.02 (Fig. S4). The specific surface area of the soil was determined as 49.0 m² 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 S2). 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 S3). 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 CO₂ measurements resulting in at least 2–3 replicates included in the final data evaluation (Table S2).

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 soil (~3.3 g dry soil at pH 2–3.6 g dry soil at pH 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 of 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, and 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 CO₂-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). CO₂ 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 CO₂ 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 β-glucosidase, β-xylodsidase, lipase, and phenoloxidase were measured using fluorometric and photometric methods (German et al. 2011; Marx et al. 2001). β-Glucosidase and β-xylodsidase 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 (β-glucosidase and β-xylodsidase). 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 (75,164) in dimethyl sulfoxide (D8418). Working solutions (1 mM) were prepared by dissolving sterile 0.1 M Tris–HCl buffer pH 6.8. Substrate solutions of β-glucosidase and β-xylodsidase were prepared and the analytical procedure was carried out following Kramer et al. (2013).

Phenoloxidase activity was photometrically measured as outlined in Ali et al. (2015) with the following slight modifications: before the measurement, we pre-incubated
the microplates at 30 °C and measured absorbance of the soil suspensions at a wavelength of 414 nm.

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 S2; Fig. S5). 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 β-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 β-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 β-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. S5).

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 & VXH-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 region 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 CO₂ (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 Zink 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), sjss 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 (Schepf 2021). Minor optical modifications (coloration) to the figures were made with Inkscape 1.0.1 (Inkscape Project 2020). Reproducible R codes and data sets generated for this study are available from Mendeley Data (https://doi.org/10.17632/2jwmgyjcr.3).

We tested significant differences between cumulative CO₂-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. S6c, Table S3).

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 S2). 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 $\beta$-glucosidase activity on MP particles because most of the data entries were below the limit of quantification (Fig. S5).

Using $p<0.05$ as the cutoff 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).

**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. 1, Table S3). PLA/PBAT particles were not mineralized in wet soil (Fig. 1, Table S3). 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. 1, Table S3). 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).

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**Fig. 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. S6c)
**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. 2, Fig. S7). 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, specific microbial respiration rates, and specific enzyme activities (Fig. 2, Table S4, Fig. S7). 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. S8c, Table S5). In dry soil, PHB addition further increased Gram-positive PLFAs and the sum of microbial PLFAs compared to the control (Table S6).

![Fig. 2](image-url)

**Fig. 2** a Sum of microbial PLFAs, b specific microbial respiration rate, and c specific β-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 β-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 effect models. The dashed line and the gray-colored area, respectively, display the mean and the 95% confidence intervals of the MP-free control soil.
We found that PHB addition stimulated the specific microbial respiration rate after 104 days, but this effect diminished after 230 days (Table S6, Fig. S8b). Specific enzyme activities were not affected by PHB addition to soil (Fig. S8a).

Regardless of MP addition, abundance and composition of the main microbial groups was different in wet compared to dry soil ($p_{F1,11} = 13.49–193.39$, $p < 0.05$) 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. S7, Table S7, Table S8). This difference was greater at 230 days for general bacterial and Gram-positive PLFAs (Table S8). Microbial PLFAs predominantly decreased from 104 to 230 days (Fig. S7, Table S7, Table S9). In contrast to this, the abundance of Gram-positive PLFAs did not change significantly in dry soil from 104 to 230 days (Fig. S7a, Table S9).

**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. 3c and g), some PLA/PBAT particles exhibited cracks in their surface structure (Fig. 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. 3i compared to Fig. 3e). In comparison, pristine MP from PLA/PBAT showed an intact and rather smooth surface (Fig. 3b). We did not observe microbial colonization on the surfaces of MP particles (Fig. 3d, f, h, and j).

**Enzyme activities on the surfaces of individual MP particles**

We measured lipase and β-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 S10). Lipase activities on the surfaces of MP particles incubated in dry soil were higher than in wet soil (Table S10). 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. S5). 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. 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 β-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 β-glucosidase activity (Fig. 4, Fig. S5). 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 β-glucosidase activity than adjacent soil (Fig. 4).

Non-incubated MP exhibited no enzyme activity. MP particles had light brown spots (Fig. S9a, 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. S9c).

**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. 2a, Table S7, Table S8, and Fig. S7).

The overestimation of PHB mineralization in wet soil (130.7%, Fig. 1) is likely due to the sudden increase in CO₂ production rates in the first 2 weeks of the experiment (Fig. S6a). 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 the 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. S8) 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. S6a 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 (Zhang et al. 2019b). 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 × 2 × 0.1 cm³) 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. S7). 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., Pseudomonadaceae, Burkholderiaceae), while restricting the growth of others.
activities on PLA/PBAT surfaces were higher than those in to our expectation, higher in dry than in wet soil. Lipase activities were enhanced compared to LDPE particles, but contrary addition, lipase activities of individual PLA/PBAT particles extracted from dry and wet soils exhibited cracks in faces would show morphological changes, PLA/PBAT particles after incubation in soil 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. 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. 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. 4). β-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 (Honest-Man 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. 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 cm²) 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 cata lyze 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. S7, Table S8), 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 are 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 (Honest-Man 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. S9) 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 (Boedinghaus 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.

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 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 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 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 microbiologically driven soil processes.

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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.

Declarations

Conflict of interest The authors declare no competing interests.

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