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Effects of microplastic and microglass particles on soil microbial community structure in an arable soil (Chernozem)

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Abstract

Since decades, microplastics and microglass enter aquatic and terrestrial environments. The complexity of the environmental impact is difficult to capture and consequences on ecosystem components e.g. such as soil microorganisms are virtually unknown. Addressing this issue, we performed an incubation experiment by adding 1% of five different types of impurities (≤ 100 µm) to an agricultural used soil (Chernozem). Four microplastic types (polypropylene (PP), low density polyethylene (LD-PE), polystyrene (PS) and polyamide12 (PA12)) and microglass were used as treatment variants. After 80 days of incubation at 20°C, we examined soil microbial community structure by using phospholipid fatty acids (PLFA) as markers for bacteria, fungi and protozoa. The results showed that soil microorganisms were not significantly affected by the presence of microplastic and microglass. However, PLFAs tend to increase in LD-PE (27%), PP (18%) and microglass (11%) treated soil in comparison with untreated soil, whereas PLFAs in PA12 (32%) and PS (11%) treated soil decreased. Interestingly, the comparison of PLFA contents between microplastic types revealed significant differences of PA12 (-87%) and PS (-42%) compared to LD-PE. Furthermore, bacterial PLFAs showed a much higher variability after microplastic incubation whereby fungi seem to be more unaffected after 80 days of incubation. Same for protozoa, which were more or less unaffected by microplastic treatment showing only minor reduction of the PLFA contents compared to control. In contrast, microglass has obviously an inhibiting effect on protozoa because PLFAs were under the limit of determination. Our study provides hints, that microplastics have, depending on type, contrary effects on soil microbiology and microglass seems to be highly toxic for protozoa.

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1. Introduction

Microplastics are used in a wide range of everyday and industrial application acting as abrasives, filler, film and binding agents. The identification and quantification of sources and pathways of microplastics to environment are highly diverse and difficult to detect. While different methods have been developed for synthetic polymer identification and quantification in sediments and water, analytical methods for soil matrices are lacking or still in an early experimental stage (e.g. Hurley et al., 2018). It is assumed that microplastics enter (agricultural) soils with soil amendments, irrigation and the use of agricultural plastic films for mulching applications, but also through flooding, atmospheric deposition and littering (Bläsing and Amelung, 2018; Hurley and Nizzetto, 2018; Kyrikou and Briassoulis, 2007; Ng et al., 2018; Weithmann et al., 2018). The extent of microplastics polluted soil ecosystems is probably much higher than previously thought. For instance, a recent study by Weithmann et al. (2018) found 895 plastic particles (> 1 mm) per kilogram and dry weight in digestate from a biowaste digester, which is used as fertilizer in agriculture after aerobic composting. Li et al. (2018) detected an average microplastic concentration of $22.7 \pm 12.1 \times 10^3$ kg^{-1} dry weight in 79 sewage sludge samples from 28 wastewater treatment plants in China. The amount of microplastics already entered soil habitats is uncertain, but Ng et al. (2018) estimated that 2.3 to 63.0 Mg ha^{-1} microplastic loadings from biosolids reached agroecosystems.

The properties of microplastics differ regarding their size, morphology, origin and chemical composition. A generally accepted definition for the term "microplastics" does not exist so far but is essential for industry, research and politics. In several studies, microplastics are defined as particles < 5 mm (5000 µm) and a contradistinction to nanoparticles is seldom given in environmental studies (Fig. 1). Most environmental studies, however, specify microplastics in large (1 mm to 5 mm) and small (1 µm to 1 mm) particles (Wagner et al., 2014). Besides a controversial debate about the term "nanoplastics" and its definition is still ongoing. Gigault et al., (2018) specified nanoplastics and recommend 1 µm as upper size limit. On the other hand, the origin of the microplastic particles plays a crucial role. The distinction between primary and secondary microplastics reveals differences between produced primary microplastics (e.g. for abrasives, cosmetic additives or industrial resin pellets) and degraded secondary microplastics, which results from formerly larger plastic debris. Due to variable formation conditions the surface properties of microplastics, which feature the same size, could be highly diverse. This circumstance leads to a varying fate and behavior of microplastics in environmental systems (Wagner et al., 2014).

There are more than 200 different types of plastic known, which have highly likely different properties e.g. regarding its reactivity or bioavailability in soil environment. For plastic differentiation, not only its size should be used for categorization in environmental research but also its chemical (e.g. hydrophobicity scales) and physical properties (e.g. morphology) which may influence physicochemical soil properties and in turn affects soil biology. A recent study by De Souza Machado et al. (2018) showed, that 2% microplastic concentration in soil affected bulk density, water holding capacity, hydraulic conductivity, soil aggregation, water stable aggregates and microbial activity. This comprehensive study elucidates the complexity of processes triggered by the presence of microplastic particles in soil environment. Microglass is currently not part of the microplastics discussion although glass is very resistant to corrosion or weathering and can be thought as corrosion-proof (Papadopoulos and Drosou, 2012). Microglass is used as blasting abrasive, filling material and an additive of road markings. It enters thus the environment on similar ways as microplastics e.g. in sewage sludge or abrasive from roads. The effects on terrestrial ecosystems are equally unknown as those of microplastics.

The present study contributes to a deeper understanding of the impact of different microplastics and microglass (~100 µm) on soil microbial community structure in an agricultural soil. For this, an arable soil and different types

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of microplastics and microglass were incubated for 80 days. In order to identify possible shifts in the microbial community structure we used phospholipid fatty analysis (PLFA). This study was guided by the following research questions:

1. Is it possible to observe distinct shifts in microbial community due to the presence of microparticles?
2. Do different plastic material properties stimulate microbial groups in diverse ways?
3. Does microglass affect the microbial community in a similar way to microplastics?

2. Material and Methods

2.1 Soil sampling and incubation experiment

Soil samples were taken on March 11, 2018 near Brachwitz (51°31'46" N, 11°52'41" E; 102 m above sea level), 10 km northwest of Halle (Saale) (Saxony-Anhalt, Germany). The samples were randomly taken at four different spots (A, B, C, D) from the first 10 cm of an arable topsoil in order to have four independent replicates, which served as basic substrate for the incubation experiment. Soil was immediately sieved (< 2 mm) after sampling. The soil samples set at a water content of 60% water holding capacity and pre-incubated for three weeks at 20°C.
A respective amount of 1% (w/w) of polypropylene (PP), low density polyethylene (LD-PE), polystyrene (PS), polyamide12 (PA12) (Rompan, Remda-Teichel, Germany) and microglass (Kraemer Pigmente GmbH & Co.KG, Aichstetten, Germany) was added to each independent soil replicate and stirred manually for homogenization. These quantity is equal to 12.6 Mg microparticles ha^{-1} (bulk density topsoil: 1.26 g cm^{-3}). This increased microplastic loads were chosen due to their already reported existence in soils near industrial areas (Fuller and Gautam, 2016). In addition, control soil replicates were incubated without additional microplastics or microglass, but due to the usage of an arable topsoil as incubation substrate it cannot be ruled out that any microplastic particles are already contaminate the basic substrate. However, in relation to the high microplastic loads added in course of the experimental design this basic entry is negligible. Incubation duration of all samples was 80 days at 20°C and was performed in laboratory bottles at dark. During this period all bottles were weekly opened to secure aerobic conditions and the total weight of each bottles was monitored. In case of weight loss, an equivalent amount of water was replenished to provide a constant water holding capacity of 60%. According to manufacturer specifications microplastics and microglass particle size range between 90-100 µm. The microplastics used in this study are commonly used in daily products and cosmetics (bottle caps, drinking straws (PP), plastic bags, milk bottles, food packaging film (LD-PE), disposable cups, packaging materials (PS), inks and clothing (PA)) and detected in high amounts in sewage sludge of Lower Saxony (Mintenig et al., 2017; Shah et al., 2008).

2.2 Soil basic properties

For soil basic characterization, soil samples were air dried and sieved (< 2 mm). Total carbon (TC) and total nitrogen (TN) analysis were carried out with a vario Max cube CNS analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany). Electrical conductivity (EC) and pH values were analyzed by using suspensions of 0.01 M $CaCl_2$ and distilled H_2O at a soil solution ratio of 1 to 2.5. Soil particle size distribution was measured in a suspension using a Helos/KR laser diffractometer (Sympatec GmbH, Clausthal-Zellerfeld, Germany) equipped with a Quixel wet dispersion unit (Sympatec GmbH, Clausthal-Zellerfeld, Germany). Before analysis the sample material was treated with a dispersing agent (0.2 M tetra-Sodium diphosphate decahydrate). For the evaluation of

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water holding capacity (WHC), 10 g of soil was weighted into a plastic cylinder with fine-mesh on the bottom and placed in water. After 24 hours, saturated samples were drained until water release stopped and weighted again for calculation of water holding capacity.

Soil chemical properties of the Chernozem topsoil (IUSS Working Group WRB, 2015) were as follows: Total organic carbon (TOC) 28.6 ± 1.8 g kg^{-1}, Total nitrogen (TN) 2.48 ± 0.13 g kg^{-1}, C:N 11.56 ± 0.15, EC 170 ± 9 µS cm^{-1} and pH_{CaCl2} 5.13 ± 0.02. Proportions of clay, silt and sand were 7.0 ± 0.2 %, 58.5 ± 3.6 % and 34.5 ± 3.7 %, respectively and the soil texture was classified as silt loam (FAO, 2006). Water holding capacity (WHC) was 0.218 ± 0.005 g_{H2O} $g_{dry\ weight}^{-1}$.

2.3 Phospholipid fatty acid analysis

For phospholipid fatty acid (PLFA) analysis, 6 g of fresh soil were extracted with a single-phase trichloromethane/methanol/citrate buffer system (1:2:0.8; v/v/v). 19:0 was added as first internal standard (IS1) to each sample for later quantification of the phospholipids. Extracts were centrifuged for 15 minutes at 4000 rpm. The supernatants were separated using a liquid-liquid extraction. Lipid fractionation was performed using a silica based solid phase extraction. Remaining phospholipid fractions of the samples and the external standards were treated by an alkaline saponification using 0.5 M sodium hydroxide in methanol followed by a methylation with boron trifluoride in methanol (12%). For separation of the PLFA methyl esters a liquid-liquid separation with saturated sodium chloride solution and hexane was used. For quality control 5-α-cholestane was added as second internal standard (IS2) after the phase separation. Analytes were transferred with isooctane into GC autosampler vials and analyzed by a GC 2010 capillary gas chromatograph (Shimadzu Ltd., Tokyo, Japan) equipped with Supelco SPB-5 fused silica capillary column (30m x 0.25 mm x 0.25 µm film thickness) and flame ionization detector. All PLFA contents were corrected for dry mass due to the use of fresh soil for extraction. For this purpose, WHC was determined subsequent to sample weighing.

Single PLFA were assigned to taxonomic groups according to following pattern: General fungi: 18:2ω6,9, 18:1ω9c, 20:1ω9c; arbuscular mycorrhizal fungi (AMF): 16:1ω5c; Protozoa: 20:4ω6c; general bacteria: 14:0, 15:0, 16:0, 17:0, 18:0; gram-positive bacteria: i14:0, a14:0, i15:0, a15:0, i16:0, a16:0, i17:0, a17:0; gram-negative bacteria: 16:1ω7c, cy17:0, 18:1ω7c, cy19:0; Actinomycetes (ACT): 10Me16:0, 10Me18:0 (Frostegård et al., 1993; Olsson et al., 1999; Zelles, 1999; Zelles et al., 1992). For total bacteria the sum of general, gram-positive, gram-negative and ACT was calculated. General and arbuscular mycorrhizal fungi were pooled in total fungi. Sum of PLFA describes the total measured content of fungal-derived and bacterial-derived PLFA.

2.4 Scanning Electron Microscopy (SEM)

Microplastic samples were fixed on an object slide and coated with gold using a Q150R ES rotary pumped sputter coater (Quorum Technologies Ltd., Laughton, United Kingdom) in a low vacuum atmosphere. The SEM images were taken with a Tabletop Microscope TM4000Plus (Hitachi Ldt., Tokyo, Japan).

2.5 Statistical analysis

Statistical analysis and graphical design were carried out using R 3.5.0 (R Core Team, 2018). Prior test assumption of normally distributed data was examined using Shapiro-Wilk test. Because of mostly non-normal distributed data Brown-Forsythe test was used for checking for homoscedasticity in the groups. Residuals of each linear model were checked graphically for homoscedasticity and normal distribution to validate the model performance.

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Because of widespread heteroscedasticity and bad model performances, differences in PLFA marker contents between treatments of each taxonomic microbial group were statistically evaluated using the Kruskal-Wallis rank sum test. Nemenyi test was performed for multiple comparison in-between one comparison group in case of a significant ($p \leq 0.05$) treatment effect in the Kruskal-Wallis test.

3. Results

3.1 Morphology and size of microparticles

The SEM images of the microplastics (PP, LD-PE, PS, PA12) and microglass are shown in Fig. 2, illustrating the heterogenic morphology between but also within the same type of microplastic. Furthermore, according the manufacturer specifications size of microplastics and microglass should range between 90 to 100 µm. Many particles are, however, much bigger (up to 200 µm) or smaller (down to 10 µm). Especially LD-PE, PA12 and PP have a slag-like structure leading to pore formation, whereas PS has a plate shaped structure with fringed or even sharp edges. Pointy and sharp edges are also shown for LD-PE, PA12 and PP. In contrast, microglass particles appear with a few exceptions more regular than the microplastic ones and could described as microspheres.

3.2 Impact of microplastics and microglass on soil microbial community structure

The total PLFA contents show no significant differences between single specific microparticles compared to the control (Fig. 3c). Nevertheless, the PLFA contents of microglass, LD-PE and PP treated soil tend to increase compared to the control by 11, 27, and 18%, respectively, whereas PA12 and PS show lower PLFA contents compared to the control by 32 and 11%. The comparisons of single plastic types show that PLFA contents of PA12 and PS are with 87% and 42%, respectively, significant lower compared to LD-PE (Fig. 3c). A similar pattern is also observable in treatment distribution of each group PLFA content of bacterial and fungi. Although, the fungi show a more inexplicit pattern compared bacteria. This might imply that a positive and negative stimulations of the single microplastics affect bacteria as well as fungi in a comparable way. Compared to the control bacteria contents showed an increase in soil treated with microglass (20%), LD-PE (33%) and PP (26%). On the other hand, decline of bacteria has been determined in soil treated with PA12 (-33%) and PS (-11%) (Fig. 3a). Total fungi PLFAs, however, show a smaller increase compared to the control by 2% (microglass), 14% (LD-PE) and 7% (PP) and a lower decrease by -20% (PA12) and -8% (PS; Fig. 3b). The treatment effect variability of bacterial-derived PLFAs are multiple times higher compared to fungal-derived PLFAs. For instance, the highest positive median deviation of total bacterial-derived PLFAs to the control is 33% (LD-PE), whereas the highest negative deviation is 33% (PA12). In contrast, positive deviation of fungal-derived PLFAs compared to the control is only 14% (LD-PE) and negative deviation is only 20% (PA12, Fig. 3a and 3b).

Regarding a whole comparison of all treatments, with the exception of protozoa, the increase of PLFA contents could be observed for all fungal (AMF and general) and bacterial (Gram-negative, Gram-positive, ACT) groups when incubated with microglass, LD-PE and PS (Fig. 4). The significant lower PLFA contents of PA12 compared to LD-PE are also shown continuously in all microbial groups (Fig. 4).

In contrast to the fairly consistent pattern of the fungi and bacteria, protozoa show a different pattern. Protozoa PLFA contents decreased for all microplastics by up to 21% (LD-PE) compared to the control (Fig. 4). PA12 and PP show a comparatively high data variability compared to the other treatments. Most interestingly, PLFA content of protozoa was under the limit of determination for all replications when incubated with microglass.

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4. Discussion

The results show, that a high amount of impurities (12.6 Mg microplastics or -glass ha^{-1}) itself do not have a significant effect on soil microbial community structure within the incubation time of 80 days. However, there is a conspicuous tendency that different types of microplastics may have promoting (LD-PE, PP) or reducing effects (PA12, PS) on soil microorganisms (Fig. 3 and 4). Furthermore, different plastics have obviously various effects on individual taxonomic groups as indicated by the significant lower values of treatment PA12 and PS compared to LD-PE (Fig. 3 and 4). As mentioned in Section 3.2, the variability of bacterial-derived PLFA are much higher than fungal-derived PLFAs, which possibly indicates that bacteria are more susceptible to interference. However, this is not surprisingly because bacteria respond relatively fast on environmental changes (e.g. changing water conditions, temperature, etc.) e.g. due to its rapid reproduction rate (e.g. Fierer et al., 2003).

Studies dealing with the impact of microplastics on soil microbiology are still lacking and, to our best knowledge, published PLFA or even DNA based studies are still missing. However, De Souza Machado et al. (2018) investigated the microbial activity after the addition of different amounts of polyester and polyacrylic fibers as well as polyethylene fragments by measuring the enzyme activity with fluorescein diacetate (FDA). The study showed that polyester and polyacrylic fibers reducing microbial activity whereas the soil incubated with polyethylene fragments showed no clear trend. The effects might be caused e.g. through changes in soil bulk density, water holding capacity or aggregate changes (de Souza Machado et al., 2018). The reasons for the observed promoting and also inhibiting effects on microorganisms from different plastic types, remain a matter of speculation and further research is necessary addressing this issues. The causes mentioned by De Souza Machado et al. (2018) are essential reasons effecting soil microbiology.

Nevertheless, the morphology and surface properties of microplastics should not be underestimated. The slag-like structure of LD-PE, PA12 and PP form wrinkles and pores (Fig. 2) may act as habitat for soil microorganisms. This in turn may have a promoting effect on the soil microbial community composition of soil as known from pore rich soil additives e.g. such as charcoal (biochar). For instance, fungal hyphae or bacteria penetrate in pores and wrinkles and are protected from predators (Lehmann et al., 2011; Thies and Rillig, 2009). Furthermore, McCormick et al., (2014) showed that microplastic particles could be act as habitat for bacteria in rivers. Umamaheswari et al. (2014) found fungi hyphae from *Penicillium sp., Fusarium sp.* and *Aspergillus sp.*, which colonized and grew on the surface of soil buried PS after 70 days. The potential colonization of microorganism on the surface of LD-PE was clearly reviewed by (Kumar Sen and Raut, 2015), who also mentioned the penetration of the microplastic surface by hyphae. In sum, LD-PE seems to benefit the bacterial and fungal colonization. Both bacteria and fungi tend to increasing populations in our experiment. LD-PE may also act as habitat as well as carbon source. The extent of these functions is mostly controlled by abiotic for example ultraviolet irradiation and temperature (Kumar Sen and Raut, 2015). Thus, the provided habitat seems to be the most important factor for enhanced PLFA in our experiment, because abiotic factors were either excluded (no ultraviolet irradiation) or kept usual (stabile temperature at 20°C). However, colonization on microplastic surfaces after incubation was not determined in this experiment and currently it is still uncertain, if colonized microplastic surface areas could also act as a hotbed for extensive soil colonization. Furthermore, it remains uncertain why PA12 seems to inhibit microorganisms in this experiment through having similar surface properties compared to e.g. LD-PE, which tends to promote the microorganisms.

Beside the morphology of microplastic, its surface chemistry has effects on soil physicochemical processes. In comparison to LD-PE, PP and PS, which show hydrophobic characteristics, PA12 combines hydrophobic and

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hydrophilic surface groups (Schmidt et al., 2015) whereby microglass has a hydrophilic surface. A study by Marangoni et al. (2018) showed, that glass microspheres (4 µm, 7-10 µm and 30-50 µm; micoglass addition of 1-5% v/v) reduced the mobility of water reflected in a large decrease of the spin-spin relaxation time of water protons, decreases in the self-diffusion coefficient of water molecules, a lower water activity, and strengthening of O-H bonds. The study further showed that glass microspheres have an inhibiting effect on *Escherichia coli* growth and the germination of *Medicago sativa* seeds. In our experiment, an inhibiting effect of microglass could not be shown for the most microorganisms with the exception of protozoa (Fig. 4). Based on the results by Marangoni et al. (2018) is highly likely, that protozoa respond in a similar way to the presence of microglass like *Escherichia coli*. According to Galloway et al. (2017), organic compounds, nutrients and pollutants can accumulate on microplastic surface in aquatic ecosystems. It can be assumed that this also occurs in terrestrial ecosystems such as soil environments. Furthermore, it is conceivable that also humic substances accumulate on microplastic surfaces leading to an increased colonization of specific microorganisms and in consequence to the formation of a bacterial biofilm. The accumulation of nutrients and water on a surface is the precondition for the formation of biofilms consisting of extracellular polymeric substances derived from bacteria (Flemming and Wingender, 2010). The formation of biofilms may occur within three weeks, as shown by Lobelle and Cunliffe (2011) investigated the surface of PE particles in marine environment. Due to the constant (water)conditions in this study, the formation of biofilms on microplastic surfaces cannot be excluded at least on LD-PE and PP particles as well as microglass indicating promoting effects on soil microorganisms reflected by increased PLFA contents.

Apart from the effects of microplastics and -glass on soil microorganism, SEM images of this study show sharp and pointed microplastic particles (Fig. 2). Due to the fact that microplastics adhere on soil organic matter, soil fauna can potentially ingest those particles. For instance, microplastic particles smaller than 50 µm were found in earthworm (*Lumbricus terrestris*) casts. The mortality rate of the earthworms increased whereas growth rate was significantly reduced (Cao et al., 2017; Huerta Lwanga et al., 2016). Zhu et al. (2018) showed, that microplastics altered gut microbiota, increases bacterial diversity of *Folsomia candida* and growth and reproduction rate of was inhibidet. Thus, sharp and fringed edges of microplastic particles may also present a serious risk of internal injuries for the soil fauna. However, microplastics do not only increase the risk of internal injuries but also inhibit the movement of *Collembola* as shown by Kim and An (2019), which could engender wide-ranging negative effects to soil faunal community.

Another important fact is the heterogeneity of microplastics. The wide variance between the several types of plastic and just as the heterogeneity of different sources prevent a generalization of scientific results. For example Cao et al. (2017) visualized polystyrene using SEM. The showed image of PS differs strongly from the plastic used in this study. So the way of producing, the pathway to environment and the degradation status of microplastics play the important role for evaluating the behavior of microplastics in soil or other environments. Furthermore, it remains ambiguous if primary microplastics added to soils causes similar effects compared to secondary microplastics, which results from the decomposition of larger plastic debris. Depending on the parent plastic material and environmental variables, highly diverse plastic surfaces could be result from an uncontrolled surface modification due to decomposition processes. This fact is already known from the comparison of primary and secondary nanoplastic properties (Gigault et al., 2018). Also the single addition of high amounts of microplastics does not reflect the ordinary way how microplastics enter an ecosystem. The accumulation of plastic particles in soils is rather a long and gradual process than a single event, which do not trigger sudden environmental impacts (Rillig et al., 2019).

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5. Conclusion

This study aimed to show, whether microplastics and -glass in soil have effects on soil microbial community structure by using PLFAs as microbial markers. The results provide hints, that already after 80 days of incubation microorganisms are either promoted or inhibited depending on the type of the impurities. Different microplastic types seem to have contrary effects on soil microorganisms depending on the origin and the properties of the plastics, which influence the morphological and chemical appearance of the microplastics. On the other hand, microglass seems to be even highly toxic for protozoa. Changes in soil microbiology induced by plastic pollution have unexpected consequences for soil ecosystems. This study should therefore be considered as basis for further research which is urgently needed in order to understand the long-term consequences of microplastics in soils and other terrestrial ecosystems.

Data availability.

All data compiled in this study is published in figures. Detailed primary data and underlying research are available by request from the corresponding author.

Author contributions.

KW conceptualized and carried out the experiment. Laboratory work was performed by KW and SP. Statistical analysis and data visualization was carried out by SP. KW prepared the manuscript with contributions from SP.

Competing interests.

The authors declare that they have no conflict of interest.

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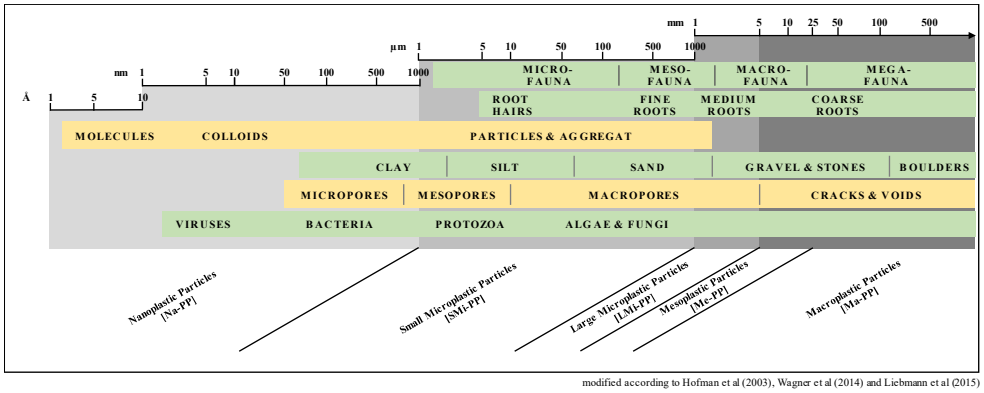

modified according to Hofman et al (2003), Wagner et al (2014) and Liebmann et al (2015)

Figure 1. Classification of plastic particles sizes in comparison with typical biotic and abiotic soil components.

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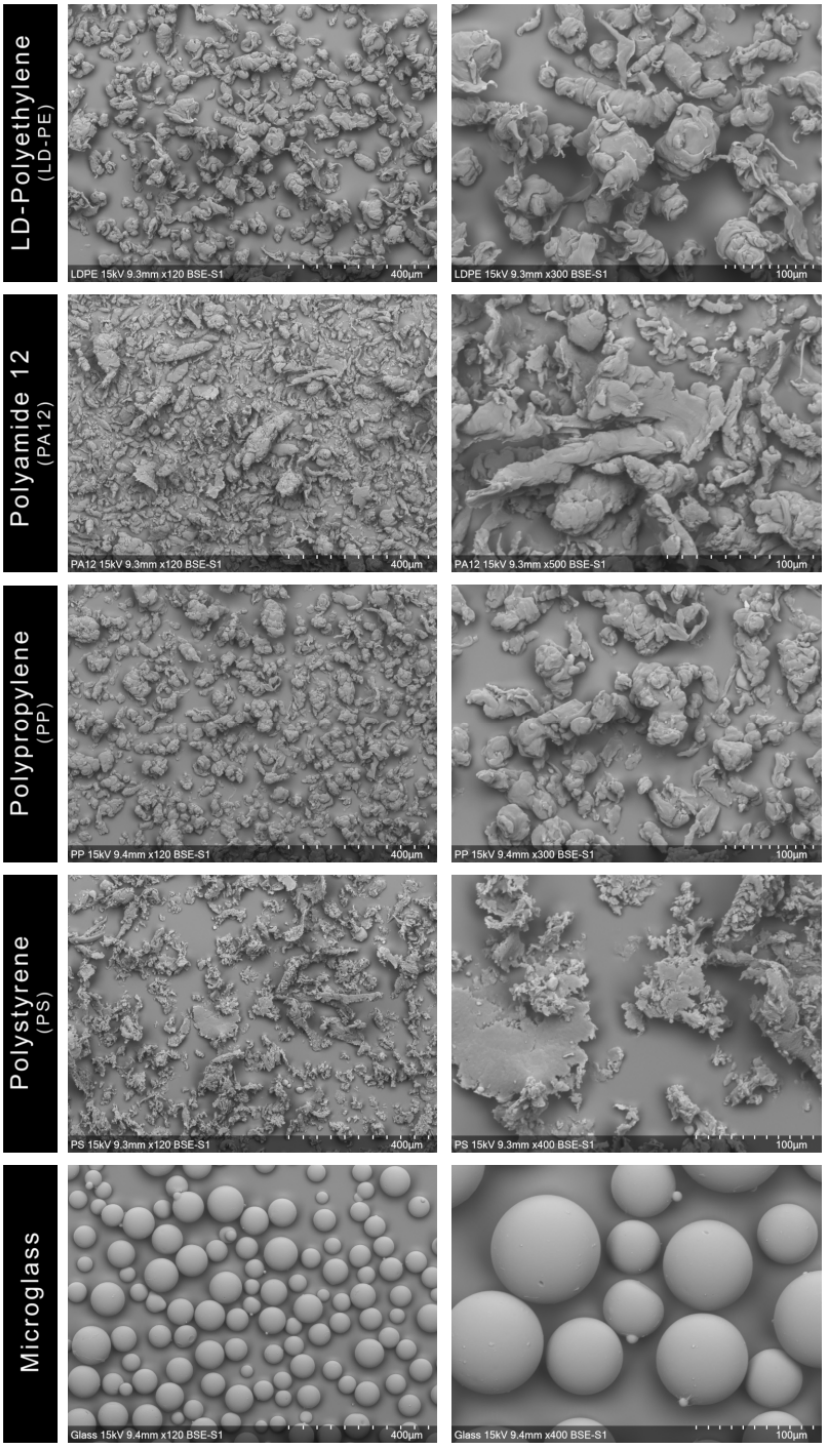

Figure 2. Heterogenic particle size distribution and morphology depending on the microparticle type visualized by SEM.

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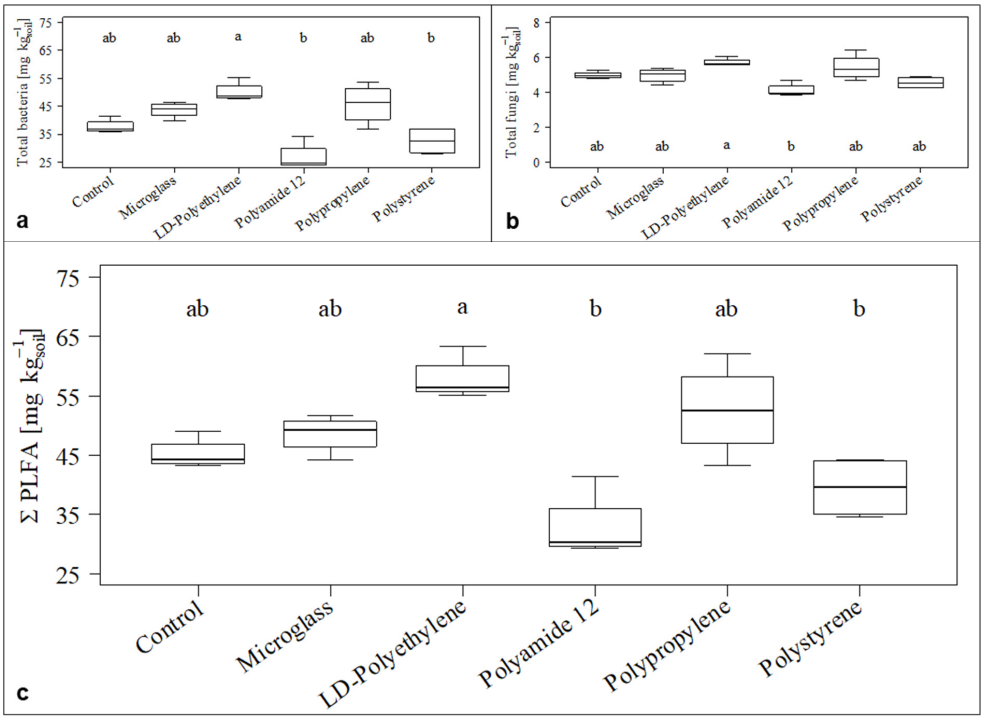

Figure 3. Phospholipid fatty acids as microbial marker in an incubated Chernozem after 80 days. a) Total bacterial-derived PLFA, b) Total fungal-derived PLFA and c) Sum of total fungal- and bacterial-derived PLFA. Different lowercases indicate significant differences according to a multiple comparison by the Nemenyi test (n=4, $p < 0.05$).

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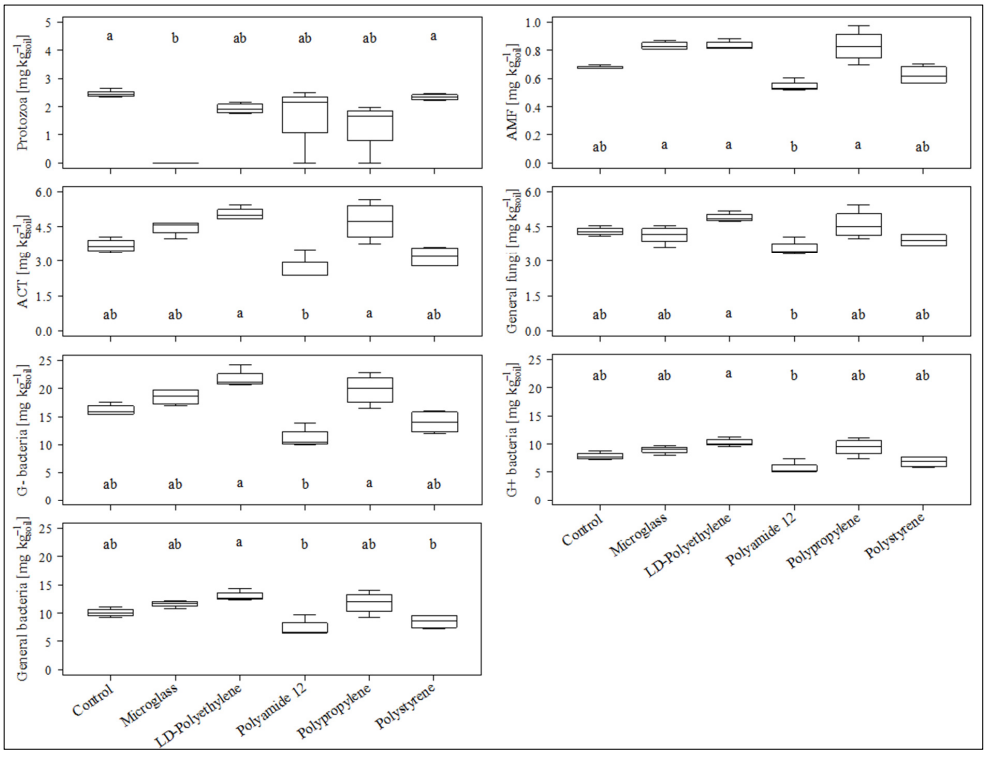

Figure 4. Microbial PLFA contents of the individual taxonomic groups of an incubated Chernozem after 80 days. Different lowercase indicates significant differences according to a multiple comparison by the Nemenyi test (n=4, $p < 0.05$). Please note the varying ordinate scales.