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Letter

Soil Storage Conditions Alter the Effects of Tire Wear Particles on Microbial Activities in Laboratory Tests

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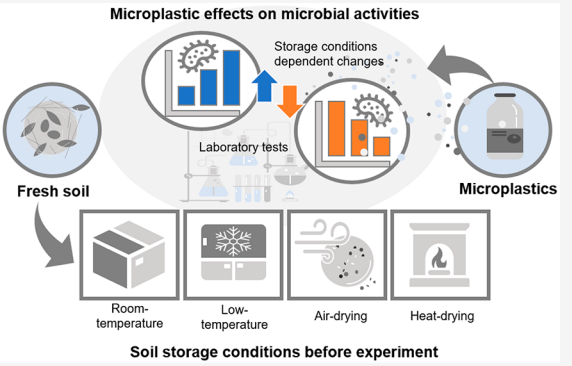
ABSTRACT: In this study, we focused on the fact that soil storage conditions in the laboratory have never been considered as a key factor potentially leading to high variation when measuring effects of microplastics on soil microbial activity. We stored field-collected soils under four different conditions [room-temperature storage, low-temperature storage (LS), air drying (AD), and heat drying] prior to the experiment. Each soil was treated with tire wear particles (TWPs), and soil microbial activities and water aggregate stability were investigated after soil incubation. As a result, microbial activities, including soil respiration and three enzyme activities (*β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, and phosphatase), were shown to depend on soil storage conditions. Soil respiration rates increased with the addition of TWPs, and the differences from the control group (no TWPs added) were more pronounced in the AD TWP treatment than in soils stored under other conditions. In contrast, phosphatase activity followed an opposing trend after the addition of TWPs. The AD soil had higher phosphatase activity after the addition of TWPs, while the LS soil had a lower level than the control group. We suggest that microplastic effects in laboratory experiments can strongly depend on soil storage conditions.



KEYWORDS: *enzymes, microbial activities, microplastic, soil respiration, soil pretreatment*

INTRODUCTION

Soil sample preparation is the first and most important step in investigating soil physicochemical and biological properties in the laboratory.[1,2] Ideally, soil samples should be immediately analyzed after field collection,[1,2] but this is difficult in practice due to the time required for transporting and processing.[3] Storage at low temperatures and air drying are generally recommended for analyzing physicochemical or biological parameters.[3−5] However, these parameters are influenced by different storage conditions and can subsequently affect soil microbial communities via drought and cold stress.[6] The release of cytoplasmic fluid from cells destroyed by the osmotic stress of drying or freezing can be accelerated after rewetting or thawing, leading to changes in microbial mineralization[7,8] and enzyme activities,[3,9] and microbial parameters such as community structure and soil respiration can be altered by storage conditions in laboratory tests.[6,9] In addition, impacts on physical parameters such as aggregate stability have been shown in previous studies.[4] A great deal of effort has been devoted to improving recommendations for storage conditions,[10] but no procedure is equally ideal for all analyses.[6]

An emerging pollutant, microplastics (<5 mm), has become a great concern worldwide. Recently, many studies have established that the effects of microplastics appear to be mediated by their physicochemical characteristics (e.g., shape, sizes, or additives),[11,12] but these results are often conflicting, in part because mechanisms underpinning microplastic effects differ from those of other typical pollutants such as metals and organic chemicals, especially in terms of microbiology.[12,13] For instance, microplastics can affect soil physical properties such as soil bulk density and water stable aggregates, which have subsequent impacts on microbial activities, community structure, metabolic rates, and other key functions.[12,13] However, these effects are still not entirely clear, at least in part due to the high variability of microplastic characteristics (e.g., types, sizes, and shapes).[12,13] Soil respiration and enzyme activities have been most often used as microbial parameters to estimate the consequences of microplastics, and many studies have shown a large variation in results, especially various enzyme activities (Tables S1 and S2). Although much effort has been devoted to understand the inconsistency of results,[13] soil storage conditions have never been discussed in microplastic research. Previous studies of microbial communities and

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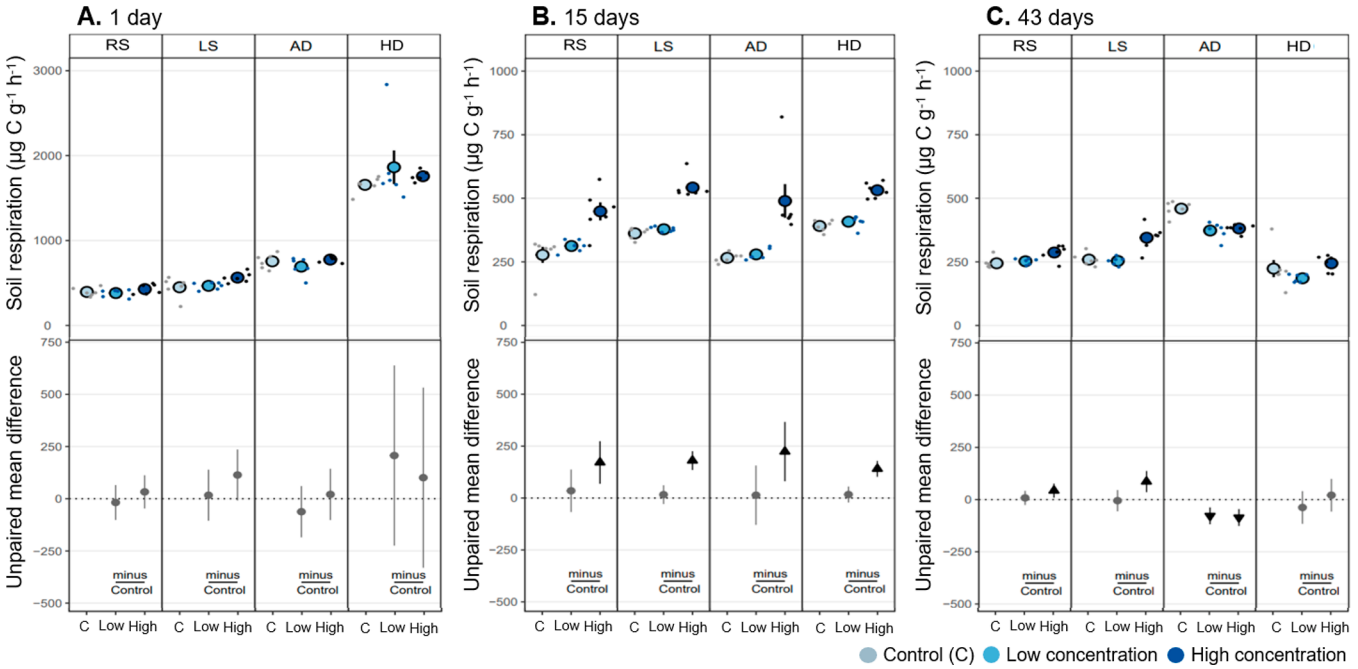

Figure 1. Effects of TWPs on soil respiration rate at different measurement times, including (A) 1, (B) 15, and (C) 43 days. Test soils were stored at room temperature (20 °C) (RS), stored at a low temperature (4 °C) (LS), air-dried at room temperature (20 °C) (AD), and heat-dried (60 °C) (HD). The top panels show the raw data distributions with the corresponding means, and the bottom panels show the unpaired mean differences after the addition of TWPs compared to the control group. Circles and triangles represent the effect size mean with the corresponding 95% confidence intervals. Negative or positive effects of each treatment compared to control groups are depicted with black triangles, while neutral effects are represented with gray circles. Note the changes in *y*-axis scaling among the panels.

enzyme activities have usually collected test soils from various fields and mostly pretreated them by drying and sieving (Tables S1 and S2). Using predried soil is popular, typically because microplastic particles can be more easily and homogeneously mixed with the dry soil, compared to the fresh (moist) soil, thus enhancing the repeatability and reproducibility of the results in laboratory tests. However, regardless of many benefits, the soil storage conditions can also alter soil physicochemical properties and the microbial community,[3,5,6] and thus, they might represent another factor that can alter microplastic effects. This unexplored question needs to be addressed to reveal the potential consequences of the soil storage conditions for microplastic experiments in the laboratory.

In this study, we tested whether soil storage conditions influence the impacts of microplastics on soil physical and microbial properties. Test soils were collected from a local grassland and stored in four different ways prior to the experiment: storage at room temperature, storage at a low temperature, air drying, and heat drying. These storage conditions are commonly used prior to the measurement of soil biochemical and microbial parameters.[6,8] We established a soil test using tire wear particles (TWPs), a specific type of microplastic, and then investigated how the effects of TWPs changed following each type of storage. We hypothesized that (1) soil storage conditions would influence the microbial properties of the test soils regardless of the addition of microplastics and (2) these changes would alter any microplastic effects.

MATERIALS AND METHODS

Tire Wear Particles. We chose TWPs as the target microplastic because they are known to be highly toxic to plant and soil fauna[14−16] and widely distributed in the soil environment.[17] Moreover, their density and shape similarities with soil particles were a benefit for mixing with fresh soils. We used the same material that has been used in previous studies, and the effects of TWPs on nematodes and plants have been previously documented.[15,16] The TWPs were prepared from a used car tire (Goodyear M+S 195/60R15 88H) using a portable belt grinding machine (Bosch PBS 75 AE) and stored at 4 °C in the dark. The average particle size was 125 μm (range of 34−265 μm), and the concentrations of heavy metals in TWPs were evaluated in a previous study (Cr, 175 ± 7 μg g^{-1}; Pb, 357 ± 29 μg g^{-1}; Zn, 5089 ± 40 μg g^{-1}; Ni, 95 ± 3 μg g^{-1}; Cu, 453 ± 10 μg g^{-1}).[16]

Soil Sampling and Storages. We collected test soil (an Albic Luvisol) from a grassland site of the Institute of Biology of Freie Universität (52.45676N, 13.30240E).[18] This soil is loamy sandy mineral soil, and the pH and carbon content are 7.1 and 1.87%, respectively. Further properties of this soil were reported in previous studies.[18,19] A large batch of this soil was stored in the greenhouse without any treatments. After 1 week, we acquired approximately 8 kg of the soil, sieved it through a 2 mm sieve, and homogenized it by hand prior to the experiment. Although these processes can be involved in the general definition of pretreatment (storage conditions), we stored the soils with four different conditions affecting in particular the soil temperature and moisture content (Figure S1). Each soil sample (approximately 2 kg) was stored at room temperature (20 °C) (RS), stored at a low temperature (4 °C) (LS), air-dried at room temperature (20 °C) (AD), or heat-dried at 60 °C for 24 h (HD). Ideally, fresh field soil should be used as a control for the comparisons of effects between each storage condition. We considered the RS soil as an alternative control soil (for storage conditions) that is the least processed

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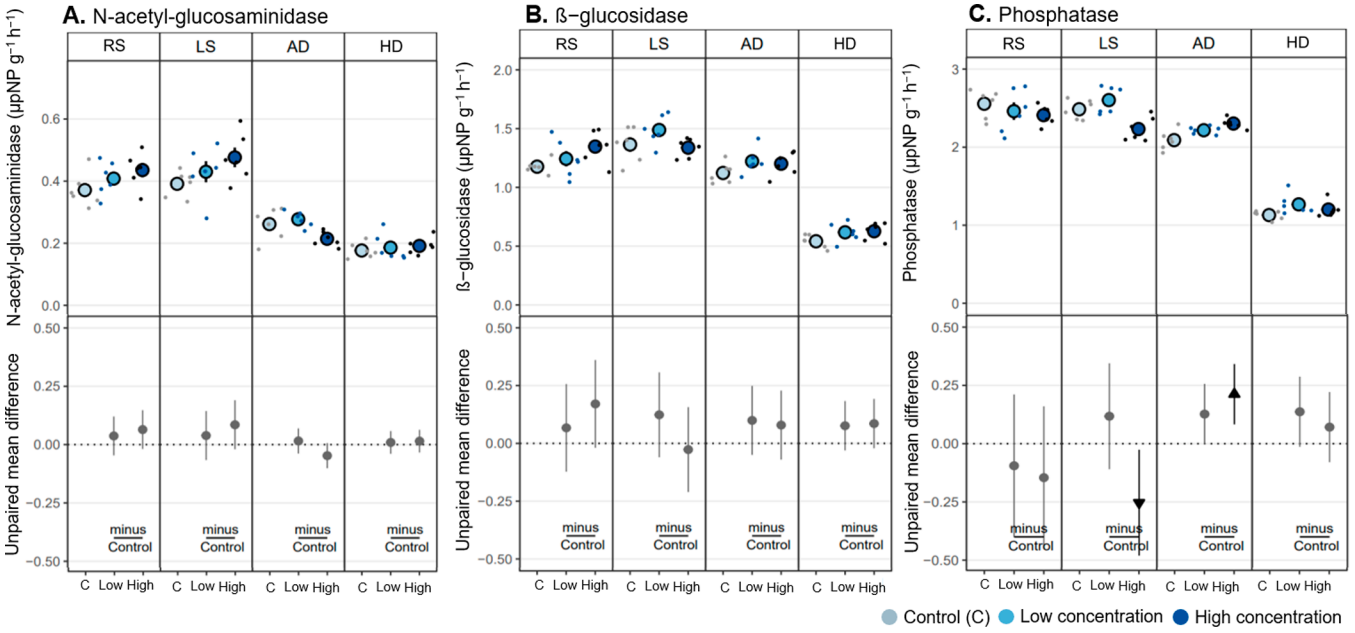

Figure 2. Effects of TWPs on soil enzyme activities, including (A) *N*-acetyl-*β*-glucosaminidase, (B) *β*-glucosidase, and (C) phosphatase. Test soils were stored at room temperature (20 °C) (RS), stored at a low temperature (4 °C) (LS), air-dried at room temperature (20 °C) (AD), and heat-dried (60 °C) (HD). The top panels show the raw data distributions with the corresponding means, and the bottom panels show the unpaired mean differences of the addition of TWPs compared to the control group. Circles and triangles represent the effect size mean with the corresponding 95% confidence intervals. Negative or positive effects of each treatment compared to control groups are depicted with black triangles, while neutral effects are represented with gray circles.

during the same storage period. Each soil was stored for 21 days, and this period was based on the data that enzyme activities can be maintained for ≤4 weeks under low-temperature storage conditions (4−6 °C).[20] The RS and LS soils were stored in a plastic box with the lid, and the boxes were covered as airtightly as possible. We opened the boxes and homogeneously mixed the soils every week. The AD soil was evenly spread and air-dried on an open tray. The HD soil was stored at room temperature (20 °C) for 20 days and heat-dried at 60 °C for 24 h. At the end of the storage period, the water contents of each soil were 11.35 ± 0.05% (RS), 11.60 ± 0.11% (LS), 0.53 ± 0.01% (AD), and 0.02 ± 0.01% (HD) (dry weight; n = 3).

Soil Incubations. We transferred 35 g of each soil into 100 mL plastic bottles (n = 6) and added deionized water to adjust the water contents according to the LS soil, which contained the highest water content (11.60 ± 0.11%). The plastic bottles were maintained at 20 °C in an incubator to ensure that the added water is evenly spread in each soil. After 24 h, each soil batch was mixed using a spatula, and a subset (30 g) of wet soil was then mixed with TWPs at two different concentrations. Test concentrations were determined to be 100 mg kg^{-1} (low concentration) and 5000 mg kg^{-1} (high concentration) based on the measurements in soil 0.5 m (400−158000 mg kg^{-1}) and 30 m (50−100 mg kg^{-1}) from roads.[21,22] The controls of each storage condition were prepared with no microplastic addition but otherwise treated the same (n = 6, total). We transferred the soil/TWP mixtures to 50 mL tubes (SARSTEDT AG & Co., Nümbrecht, Germany) and covered them with a vented cap. Tubes were incubated in a 20 °C laboratory incubator (PP110plus, Memmert GmbH, Schwabach, Germany) in the dark for 43 days. The water contents in each tube were checked and replenished with deionized water every week to keep uniform moisture during the incubation period. We measured soil respiration, enzyme activities (*β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, and phosphatase), and water stable aggregate of each treatment. The experimental procedures for each parameter are described in the Supporting Information.

Statistical Analysis. All statistical analyses were performed with R version 4.0.2 (R Core Team, 2020). The sizes of the effect (measured data of the experimental effect) of TWP treatments (two concentrations) under four different storage conditions were tested with a two-step method. First, the package "dabestr" was used to generate unpaired mean differences and a 95% confidence interval (CI) by a bootstrapping approach (5000 iterations).[23] All confidence intervals are by default bias-corrected. This approach focuses on the effect size and its precision and can avoid the pitfalls of significance testing. Second, one-way analysis of variance (ANOVA) followed by a TukeyHSD test was implemented to compare each treatment with the control. The graphs were made using the graphic package "ggplot2".[24]

RESULTS AND DISCUSSION

Soil Respiration. Soil respiration was influenced by different soil storage conditions and the addition of TWPs (Figure 1 and Figure S2). Because the RS soil was the least processed during the same storage period, we considered the RS soil as a control-like treatment for the comparison between storage conditions. One day after soil incubation and in the absence of TWPs, the soil respiration rate of each soil increased to 114 ± 31% (LS), 191 ± 22% (AD), and 419 ± 24% (HD) compared to that of the RS soil, and these differences were diminished at the end of the experiment (Figure 1A). The soil respiration rates of all treatments decreased with incubation time, and the TWP treatments showed higher soil respiration rates during the early stage of incubation (days 8−29) compared to the controls (Figure S2).

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Fifteen days after soil incubation, the respiration rates of each group were 278 ± 77 (control), 315 ± 24 (low), and 449 ± 87 (high) μg of C g^{-1} h^{-1} for RS soil, 362 ± 22 (control), 378 ± 10 (low), and 542 ± 46 (high) μg of C g^{-1} h^{-1} for LS soil, 266 ± 19 (control), 279 ± 22 (low), and 489 ± 162 (high) μg of C g^{-1} h^{-1} for AD soil, and 391 ± 22 (control), 408 ± 24 (low), and 532 ± 31 (high) μg of C g^{-1} h^{-1} for HD soil (Figure 1B). The increase or decrease in soil respiration in each treatment was observed 36−43 days after soil incubation, but there was no clear trend depending on the addition of TWPs (Figure 1C and Figure S2).

The soil respiration rates decreased over time for all treatments, and this may be related to the reduction of available labile substrates in the soils.[25,26] The increase in soil respiration rates after drying and rewetting, which is termed the Birch effect,[27] has been found in many studies[6] and can be observed within 1−11 days of rewetting.[7,28] In this study, the predried soils (AD and HD) on day 1 showed much higher soil respiration rates (755 ± 86 and 1656 ± 93 μg of C g^{-1} h^{-1}, respectively) compared to those of the RS and LS soils (395 ± 49 and 450 ± 121 μg of C g^{-1} h^{-1}, respectively), which preserve the initial field moisture content, and the respiration rates of AD and HD soils decreased on days 8 and 15 while the RS and LS soils showed a more gentle slope (Figure 1 and Figure S2). There is uncertainty about the mechanisms underpinning the CO_2 pulse after drying and rewetting, but several studies have proposed that it would be a result of mineralization of non-biomass organic matter or from the carbon from microorganisms.[7,29]

We found that enzyme activities decreased in the predried soils (AD and HD), compared to those of the RS and LS soils (Figure 2). Previous studies have reported that the temperature at which the soil is stored (4 or 20 °C) has a minimal influence on enzyme activities,[3,9] while soil hydrolases are more likely influenced by drying and rewetting.[5] Microplastics can alter soil enzyme activities via alteration of soil physicochemical characteristics or by the release of chemical additives,[13,26] but results can vary depending on the properties of soils and microplastics used (Table S2). Many studies have reported that microplastics can influence soil physical properties, leading to changes in microbial activities.[12,13] Our study was among the first to assess the effects of TWPs on soil enzyme activities, and phosphatase was the only enzyme that showed a significant difference from the control group (Figure 2C). However, a change in soil structure did not seem to contribute to the effects of enzyme activities because we observed no significant differences in water stable aggregates (Figure S3).

The effects of microplastics on soil respiration generally depend on their shapes and compositions.[26,30] For example, polypropylene fragments inhibit soil respiration, while polyethylene terephthalate film, low-density polyethylene, polyethylene foams, and biodegradable poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) can promote soil respiration.[26,30,31] The addition of TWPs has been observed to increase the rates of soil respiration,[16] and several studies have proposed potential mechanisms, such as the release of chemical additives from TWPs and the increase in pH.[16,17] The differences between the storage conditions and the control were especially evident early in the incubation period. These differences were prominent in the predried soils, especially AD soil, and may be linked with a higher initial soil respiration rate due to the Birch effect. Although the HD soil showed the strongest Birch effect, microbial activity was not stimulated, and it can be supported by relatively lower enzyme activities when soil was heat-dried (Figure 2).

Enzyme Activities. We measured soil enzyme activities at the end of the incubation period and found that soil enzyme activities can be changed depending on different storage conditions. The enzyme activities in the HD soil were 52−56% lower than in the RS soil, while the LS soil showed similar levels in each enzyme activity (RS = LS > AD > HD). The *N*-acetyl-β-glucosaminidase and phosphatase activities in the AD soil decreased to 70% and 82% compared to the RS soil, respectively. No significant effect of TWPs was found in *N*-acetyl-β-glucosaminidase and β-glucosidase activities (Figure 2A,B), while the phosphatase activity in the LS and AD soils showed an opposing trend after the addition of TWPs (Figure 2C). In the LS soil, the phosphatase activity was 2.49 ± 0.12 (control), 2.60 ± 0.17 (low), and 2.23 ± 0.15 (high) μpNP g^{-1} h^{-1}, while those in the AD soil were 2.09 ± 0.13 (control), 2.22 ± 0.05 (low), and 2.30 ± 0.06 (high) μpNP g^{-1} h^{-1}.

The RS soil, which is the least processed and most environmentally relevant soil, showed no significant changes in enzyme activities. However, the AD soil showed higher phosphatase activity after the addition of TWPs, while the LS soil had lower levels compared to the control group. Some microorganisms such as Gram-positive bacteria can tolerate drought due to their peptidoglycan content in the cell wall,[32] but other groups that cannot tolerate such stresses may enter a dormant state, form spores (for example, fungi), or die.[33] Although microorganisms that have frequently experienced drying and rewetting cycles can be resistant to drought stress, the microbial community structure can still be altered.[7,32] The predominant Gram-negative taxa in the soil can be substituted with Actinobacteria and other Gram-positive taxa,[34] and the Gram-positive:Gram-negative ratio generally increases with drought stress.[35] Members of the Gram-positive group are more active in decomposition of persistent compounds than members of the Gram-negative group,[36−38] and it may cause higher enzyme activities in the AD TWP treatment because the microplastics (including TWPs) are often considered as a presumably persistent carbon pool.[17,39] These changes were not observed during the low-TWP concentration treatment or in the HD soil containing the lowest microbial activity. However, this trend could be linked with the previous study reporting that short incubation of rewetted soil samples can induce larger fluctuations in phosphatase activity, compared to field-moist soils.[4] The release of chemical additives from TWPs has been suggested as an important toxicity mechanism.[16,17] Previous studies have reported that microbial community diversity and structure are not significantly influenced by various storage temperatures (−80 to 20 °C),[40] but the properties of the individual cells such as membrane fluidity and proteomic profiling can be changed after cold stress,[41] potentially influencing the bioavailability and toxicity of the chemical additives from TWPs.

Soil Water Stable Aggregates. The water stable aggregates were changed depending on different storage conditions (Figure S3). The water stable aggregates in the AD soil were 23−41% lower than those in the RS soil, while the LS soil showed the highest level. However, no significant effect of TWPs was found in each treatment.

Soil Storage Conditions and Potential Microplastic Effects. Our study highlights that the effects of the addition of TWPs on microbial activities can depend on the storage

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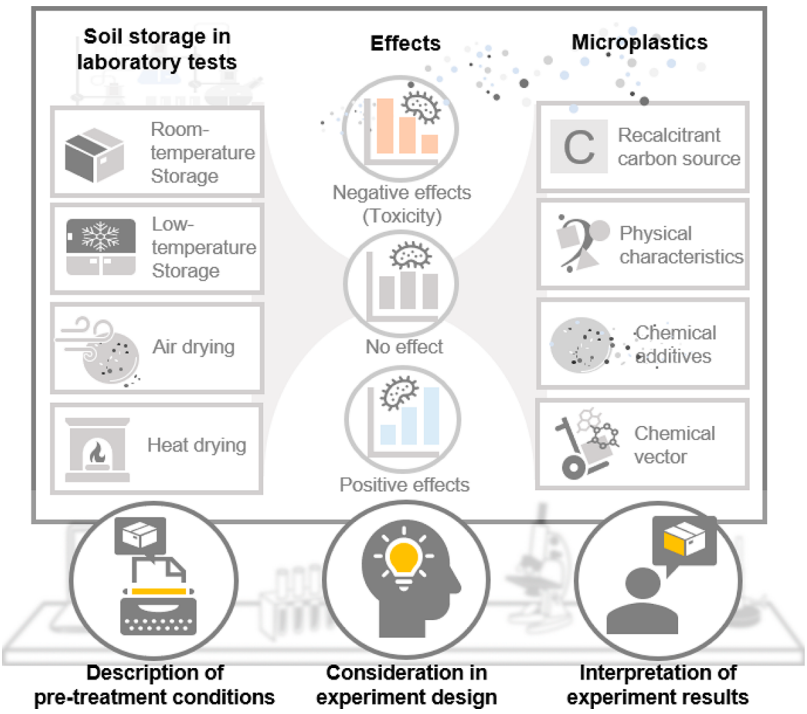

Figure 3. Conceptual depiction of the impacts of soil storage conditions and consequences for the interpretation of results.

conditions of soil and subsequently result in varying responses on soil processes. This finding suggests that, given the range of storage conditions that are routinely used (Tables S1 and S2) in microplastic work, a significant proportion of the variability in the results of the addition of microplastics to soils could be due to this aspect.

Microplastics represent a ubiquitous pollutant suite, with direct and indirect impacts through multiple mechanisms.[11,12] The effects of microplastics on soil microbial activities have been revealed in many studies, but often their results are conflicting.[13] These differences in results are mostly suspected to be due to various characteristics of microplastics.[26] In addition, the effects of microplastics on microbial communities are often distinct between different soil types,[42−44] and several studies have reported that the soil property-dependent changes may be more important than the characteristics of microplastics.[42] Despite the impact of storage conditions, such as those observed in this study, many studies to this date do not clarify the storage conditions prior to experiments (Tables S1 and S2). Our findings indicate that soil storage conditions may represent another key factor that can shift potential microplastic effects, and this potential variable should be considered an important parameter in future studies that investigate the effects of microplastics in the soil system. It is thus imperative that soil storage conditions be considered during the process of experimental design and in the subsequent interpretation of results (Figure 3). In this study, we found that the soil storage conditions can contribute to the changes in the effects of microplastics on microbial activities. This issue is important because any observed effects after the soil storage conditions in the laboratory might mean that the true extent of microplastic effects has been over- or underestimated in many lab-based studies. The alterations in microplastic effects by soil storage conditions are important for understanding differences between field and laboratory experiments on microplastics and likely are also relevant for other types of microplastics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.2c00585.

Supplementary methods, supplementary figure of the diagram of soil tests with four different storage conditions (Figure S1), soil respiration (Figure S2), effects of TWPs on water stable aggregates (Figure S3), and lists of previous studies (Tables S1 and S2) (PDF)

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Author Contributions

S.W.K.: conceptualization, design of the study, experiment setup, analysis of data, and writing. Y.X.: design of the study, experiment setup, analysis of data, and writing. P.M., M.B., and Y.Z.: design of the study, experiment setup, and writing. M.C.R.: review and editing. All authors contributed to the article and approved the accepted version.

Notes

The authors declare no competing financial interest.

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