Microplastics and Nanoplastics

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Seedling development of perennial ryegrass is impacted by polyvinylchloride microplastics, tire wear particles and their leachates

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Abstract

Increasing amounts of microplastic (MP) in soils may pose a hazard to agricultural ecosystems. To date, research has largely concentrated on MP quantification in environmental samples and MP impacts on aquatic organisms, humans and food chains, whereas responses of crop and fodder plants to MP particles and their respective leachates remain a knowledge gap. The present study evaluated the effects of two MP types - polyvinylchloride (PVC) and recycled tire crumb (RTC; proxy for tire wear particles, TWP) - and their respective leachates on germination and early growth of *Lolium multiflorum* in a laboratory trial. For fragment treatments, different concentrations and RTC size fractions were applied, whereas leachate treatments were applied at a single extract ratio, to disentangle chemical from physical MP effects. Leaf δ^{13}C-signatures were additionally measured as a proxy for early physiological responses. Exposure to fragments or leachates reduced normal germination and increased the proportion of ungerminated and abnormally germinated seeds. Leachates predominantly affected germination and root elongation, but not biomass production; the strongest effects were observed for PVC leachates. Fragments generally caused stronger adverse effects than leachates, and PVC affected growth parameters more than RTC. Contrary to expectations, the smallest RTC size did not induce the strongest responses, and no consistent interaction between RTC fragment size and concentration was observed. Leaf δ13C-signatures showed treatment-related trends, but values were depleted rather than enriched, consistent with altered carbon isotope discrimination and early physiological functioning during seedling growth. This study focused on comparatively large concentrations of MP; therefore, our results should be interpreted in terms of hazard potential rather than as a direct representation of MP loads under field conditions. Overall, the findings confirm that MP can impair plant establishment and affect early physiological processes. Chemical (leachate-mediated) and physical (fragment-mediated) effects are both relevant in this context.

Keywords Microplastics, Germination trial, Tire wear, Environmental leaching, Carbon isotope signatures/ δ^{13}C

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Introduction

All-sized plastics, including microplastic (MP; < 5 mm) have been identified as a potential stressor for terrestrial ecology, plant-soil-systems and multiple associated organisms [1–3]. Thermoplastics such as polyvinylchloride (PVC) are frequently detected in environmental samples [4]. Additionally, tire-derived particles are an important source of MP-associated environmental emissions [5, 6]. These particles are emitted as a result of abrasion and friction between tire tread and road surfaces and therefore highly heterogeneous [5, 7, 8]. In this study, we considered recycled tire crumb (RTC) as a proxy for tire wear particles (TWP), meanwhile acknowledging that RTC differs from environmentally emitted TWP.

MPs are generally classified as primary MP, which are deliberately manufactured and typically defined with an upper size limitation of 5 mm [9, 10], whereas secondary MPs originate from physical, chemical or biological degradation of larger plastics [11]. Photo-aging, water and wind erosion as well as thermal oxidation contribute to fragmentation and facilitate the widespread dispersal of these particles [12].

Agricultural soils and grasslands are considered major sinks for MP, due to inputs from agriplastics and organic amendments such as sewage sludge, composts and digestates [13–16]. TWP and other MPs may also be deposited onto soils via atmospheric transport, run-off or precipitation [17, 18], thereby contributing to diffuse contamination beyond direct source areas [18]. These entry pathways introduce MPs not only as particles but also as sources of specific leachable compounds that may be mobilized when contacting water and therefore cause other effects than the parent particles do. Because properties of MP are highly variable, the range of effects so far reported for terrestrial ecosystems and plants is large and heterogeneous [9, 19, 20].

The increasing prevalence of TWP has led to experiments revealing TWP to be harmful for soil health, plants and soil organisms [21–23]. For example, exposure to TWP has been demonstrated to reduce shoot and root growth of *Allium porrum* [21]. Still, the effects of TWP on soil and plant metabolism and the underlying mechanisms of action are not yet understood [24]. To date, plant-related MP research is still scarce [25, 26] and has focused on major crops such as wheat [27], lettuce [28] and rice [29] with reported effects from positive to toxic, depending on polymer properties and experimental endpoint [30–33]. Considerably fewer studies have investigated the effects of MP on fodder plants despite the major role that grasslands play in global livestock feeding [34]. MP effects on grassland species therefore deserve particular attention [35]. Esterhuizen et al.. investigated the effects of polyethylene MP and its leachates on *Lolium multiflorum* and revealed germination and general plant growth being adversely affected [33]. Similar findings were published by Boots et al.. who exposed *Lolium perenne* to polylactic acid and polyethylene MP and observed reductions in germination rate and shoot height [30].

Germination and early seedling development are pivotal stages that determine establishment success and population density [36, 37] and may therefore be specifically sensitive to MP exposure [20, 38, 39]. Beside germination, root elongation is another key parameter for plant growth performance and MP clogging and adsorption onto root hairs may lead to reductions of root growth [40–42]. Development of functional root systems is closely linked to maintenance of a steady plant water balance. However, so far, the potential effects of MP on water balance and water use of grassland species have not been studied.

Leaf $\delta^{13}C$-signatures can serve as a suitable indicator for physiological functioning linked to stomatal regulation and carbon assimilation; in this context, $\delta^{13}C$ can be interpreted as a composite proxy rather than a single key indicator of water stress. In MP research, stable isotope approaches have predominantly been used for polymer characterization and tracing of nutrients, but have rarely been applied to assess plant-MP-interactions [43–45].

Physically intact plastics are considered to be biochemically inert [46], but additives that are frequently not bound to polymers are likely to leach from the plastics during surface weathering and aging processes [46–48]. These leachable compounds may cause effects that differ from those induced by the parent molecule, depending on polymer type and exposure conditions [19, 49]. For instance, TWP leachates contain high amounts of heavy metals (e.g. Zn) and organic compounds (benzothiazoles) [24]. Chemically induced leachate effects are one possible facet of how MPs may impact plant growth. In parallel, physical effects of MP particles may come from adherence to the seedling's surface, initiating physical root entanglement and alterations in seed hydration and root surface structures [40, 45, 50–53].

Overall, the available evidence suggests that MPs may harm plants through both chemical (leachate-mediated) and physical (particle-mediated, i.e., fragment) pathways. Despite the relevance of these pathways, their interplay and relative contributions remain poorly understood for PVC and tire-derived particles (RTC) in fodder species.

Therefore, we studied the effects of these particles and their respective leachates on the germination and early plant growth of *Lolium multiflorum* under controlled laboratory conditions. We tested different concentrations and RTC size fractions to disentangle chemical (leachate-mediated) from physical (particle-mediated) effects. To clarify these potential toxicity reactions and threshold effects during early seedling development, we used

a wide concentration gradient including comparatively high fragment loads. These loads were applied in a filter-paper-based setup, reflecting high-dose contact exposures that are not directly comparable to environmental soil concentrations (mg kg^{-1}). Leaf $\delta^{13}C$-signatures were measured as an indicator of alterations in physiological processes during initial growth stages, including stomatal regulation, photosynthetic performance and carbon assimilation. We hypothesized that (a) exposure to MP fragments (PVC and RTC) impairs germination rate and early growth (biomass; total root length); (b) the magnitude of these effects is modulated by fragment concentration and size (for RTC), with stronger responses expected at higher concentrations and smaller fragment sizes; (c) fragments and leachates elicit different responses: leachates predominantly affect germination and total root length, whereas fragment exposure inhibits growth via physical interference; and (d) MP fragment and leachate exposure results in different leaf $\delta^{13}C$-signatures, reflecting alterations in early physiological processes.

Materials and methods

Experimental design and setup

The tested factors were type of MP (PVC vs. RTC size fractions W1-W3), concentration level (0.5, 1.0, 1.5, 2.0 g per Petri dish for fragment treatments only), and application type (fragment vs. leachate) in a randomized block design (three spatial blocks), including controls without MP. Controls were replicated once per block ($n = 3$ in total), the design was therefore not orthogonal, so the control samples ranged outside the factorial setting. In total, $N = 63$ Petri dishes were used ($n = 48$ fragment treatments, $n = 12$ leachate treatments, $n = 3$ controls).

MP fragment loads were: 0.5 g; 1.0 g; 1.5 g and 2.0 g per Petri dish (approximately 5–21 mg/cm^2), representing a deliberately wide gradient of concentration. Glass petri dishes (11 cm in diameter) were lined with moistened filter paper (Whatman® No. 11). The corresponding quantity of MP was applied as a surface load onto the filter paper and 50 seeds each of *Lolium multiflorum* Lam. var. *westerwoldicum* (var. "Pollanum", DSV-Deutsche Saatveredelung, Lippstadt, Germany) were evenly distributed. Only seeds of physical integrity were included. Fragments or corresponding leachates were added and dishes covered with glass lids. Throughout the experimental period, moisture inside the Petri dishes was maintained by daily watering (1 ml), either with demineralized water (fragments/controls) or leachate. This germination trial represents direct particle-seed-contact in a static exposure system rather than an isolated suspension-based setup. Experiments were conducted for 13 days under laboratory conditions (mean temperature of 21 °C) using neutral white growth bulbs (Growlight Duo, Parus, Michelstadt, Germany) for a 12-h cycle of alternating dark/light.

Plastic materials and leachates

Two types of MP were used in the experiment: PVC (Werthmetall, Grammetal, Germany) as a powder in a size range 1–63 µm and RTC (MRH Muelsen, Muelsen, Germany) used as a proxy of TWP, following Luo et al. [54]. Three size ranges of RTC were chosen: W1 (2000–5000 µm), W2 (600–1600 µm) and W3 (0–400 µm). All these RTCs were of irregular shape due to the shredding process.

Leachates were prepared at a single extraction ratio without a concentration gradient (1:10; 2 g material in 20 mL demineralized water) for PVC and each RTC size fraction. Mixtures were kept in glass sample tubes for seven days at room temperature with daily stirring and were subsequently filtered using ROTILABO round filters (retention 2–3 µm). Leachates were operationally defined as aqueous extracts filtered to 2–3 µm. The filtrates were stored at room temperature in Erlenmeyer flasks (sealed with Parafilm) until use. Leachates were not chemically characterized in this study. Therefore, PVC- and RTC-derived additives were not quantified and their contribution to the observed effect patterns could not be assessed.

Measurements

The state of germination was recorded daily, with radicle emergence considered as the visible sign of germination. At the end of the experimental period, seedlings were gently rinsed off adhesive MP and separated into normally germinated seedlings, abnormally germinated seedlings or non-germinated seeds, following the ISTA-handbook of seedling evaluation [55]. Abnormal germination was assessed as proportion per Petri dish (abnormal seedlings / 50 seeds). Seedlings were classified as abnormal, when there was (i) no or only short development of primary roots while the plumule appeared normal; (ii) where primary roots developed without corresponding development of the shoot/plumule and (iii) in cases of malformed root tips (e.g. deformed/twisted roots) [56].

Root and shoot biomass of the seedlings were analysed separately. The total root length per Petri dish was analysed via root scanning. Roots were prepared on transparent PP films to avoid overlapping and scanned at 600 dpi (DCPL8400CDN, Brother, Bad Vilbel, Germany). After scanning, images were converted to 8-bit, contrast was adjusted uniformly and all non-root artefacts were removed from the image. Root length was determined in ImageJ (v1.53 e) using the Ridge Detection plugin, which summed the root length (cm) per dish.

For measurement of carbon isotope signatures ($\delta^{13}C$) of dried shoots, aliquots of shoot material (0.4 mg) were weighed, enclosed into tin capsules in duplicates and combusted at 1050 °C in an elemental analyser (EA; Vario PYRO Cube®, Elementar, Langenselbold, Germany) coupled to an Isoprime 100 isotope ratio mass spectrometer (IRMS, Elementar, Langenselbold, Germany). Accuracy was checked by including the internal laboratory standard in every run (rye flour), calibrated against international secondary standards from the International Atomic Energy Association (IAEA): NBS-18 (calcite) and IAEA-600 (caffeine) for ^{13}C. These were calibrated against the standard VPDB (Vienna Peedee belemnite).

The isotope signatures are reported by using the delta notation:

$$\delta^{13}C\,[‰] = [(^{13}C/^{12}C_{sample} - ^{13}C/^{12}C_{standard})/^{13}C/^{12}C_{standard}] \times 1000,$$

where $^{13}C/^{12}C_{standard}$ is the measured ratio of the reference standard and $^{13}C/^{12}C_{sample}$ is the isotope ratio of the sample. The internal reproducibility of the IRMS system was 0.07‰ over the lifetime of the used ion source.

Statistical analysis

All statistical analyses were conducted in the R environment [57]. Data were screened for normality using the Shapiro-Wilk-test and for homogeneity of variances using Levene's test (package car [58]) and statistical significance was accepted at $p < 0.05$. Fragment and leachate treatments were analysed separately. Associations between $\delta^{13}C$ and growth responses (fresh weight and total root length) were assessed using Spearman's rank correlation, for both fragments and leachates - the controls were excluded here.

The effect of MP treatment (MP type and, for RTC fragments, size fraction) and where applicable, MP concentration, was analysed using one-way and two-way analyses of variance (ANOVA) for each response variable. Effect sizes for the ANOVA were reported as eta squared (η^2), where appropriate. For normally distributed parameters, Tukey's HSD pairwise comparisons were used as post-hoc tests to detect significantly different means ($p < 0.05$). Where appropriate, data were transformed to better meet ANOVA assumptions; otherwise, non-parametric Kruskal–Wallis tests were applied followed by Dunn's post-hoc tests with Bonferroni correction. Post-hoc p-values were adjusted by either Tukey's HSD or Bonferroni correction, depending on the analysis. Non-parametric tests (Kruskal-Wallis/Dunn) were obtained from the rstatix package [59]. For the Kruskal–Wallis tests applied, effect sizes were reported as epsilon squared (ε^2). All results are reported as means ± SEM (standard error of the mean), unless otherwise indicated. The package ggplot2 [60] was used for graph creation.

Results and discussion

Hypothesis A: MP impairs germination and early plant growth

In line with our first hypothesis, we recorded an overall negative impact of MP fragments in the germination environment of ryegrass: Kruskal–Wallis tests indicated significant differences among treatments on normal germination ($p < 0.001$; $\varepsilon^2 = 0.588$). Dunn's post-hoc tests with Bonferroni adjustment showed that PVC fragments significantly lowered normal germination when compared to the control ($p_adj = 1.10 \times 10^{-4}$), whereas those with RTC fragments did not differ significantly from the control ($p_adj = 0.273$) (Fig. 1A). Although the overall Kruskal-Wallis test on abnormal germination was significant ($p = 0.002$; $\varepsilon^2 = 0.216$), the differences between PVC or RTC and the control were not significant in the Dunn post-hoc test after the Bonferroni adjustment (PVC: $p_adj = 1.00$; RTC: $p_adj = 0.284$) (Fig. 1B).

Impaired germination may cause inhibitions of further plant growth. Fresh weight was highest for the control plants (0.80 ± 0.33 g), followed by RTC (0.42 ± 0.03 g) and lowest for PVC treatments (0.03 ± 0.01 g). Kruskal–Wallis tests indicated significant differences among treatments for fresh weight ($p < 0.001$; $\varepsilon^2 = 0.534$). Dunn's post-hoc tests (Bonferroni) showed that PVC fragments significantly reduced fresh biomass when compared to the control ($p_adj = 0.00118$), whereas RTC fragments did not significantly differ from the control samples ($p_adj = 0.867$) (Fig. 1C).

MP fragments also impacted root elongation. Longest roots were generated within the control group (374.01 ± 38.5 cm), followed by RTC treatments (183.89 ± 14.0 cm), whereas the shortest roots were recorded for PVC treatments (0.34 ± 0.21 cm) (Fig. 1D). Kruskal–Wallis tests indicated significant differences among treatments on total root length ($p < 0.001$; $\varepsilon^2 = 0.613$). Dunn's post-hoc tests (Bonferroni) showed that PVC fragments significantly reduced total root length in comparison to the control ($p_adj = 2.69 \times 10^{-5}$), whereas RTC fragments did not cause significant effects compared to the control ($p_adj = 0.116$).

Our results are in line with various studies finding negative effects of MP on germination and growth [61–64]. Besides physical clogging that may cause the impairment, chemical effects are also probable: Wang et al. revealed ecotoxicity of PVC, displayed in root growth decline of wheat by 3.56–7.45% [65]. Colzi et al. [61]. observed reduced root development of *Cucurbita pepo L.* especially in the presence of PVC MP, as this polymer is

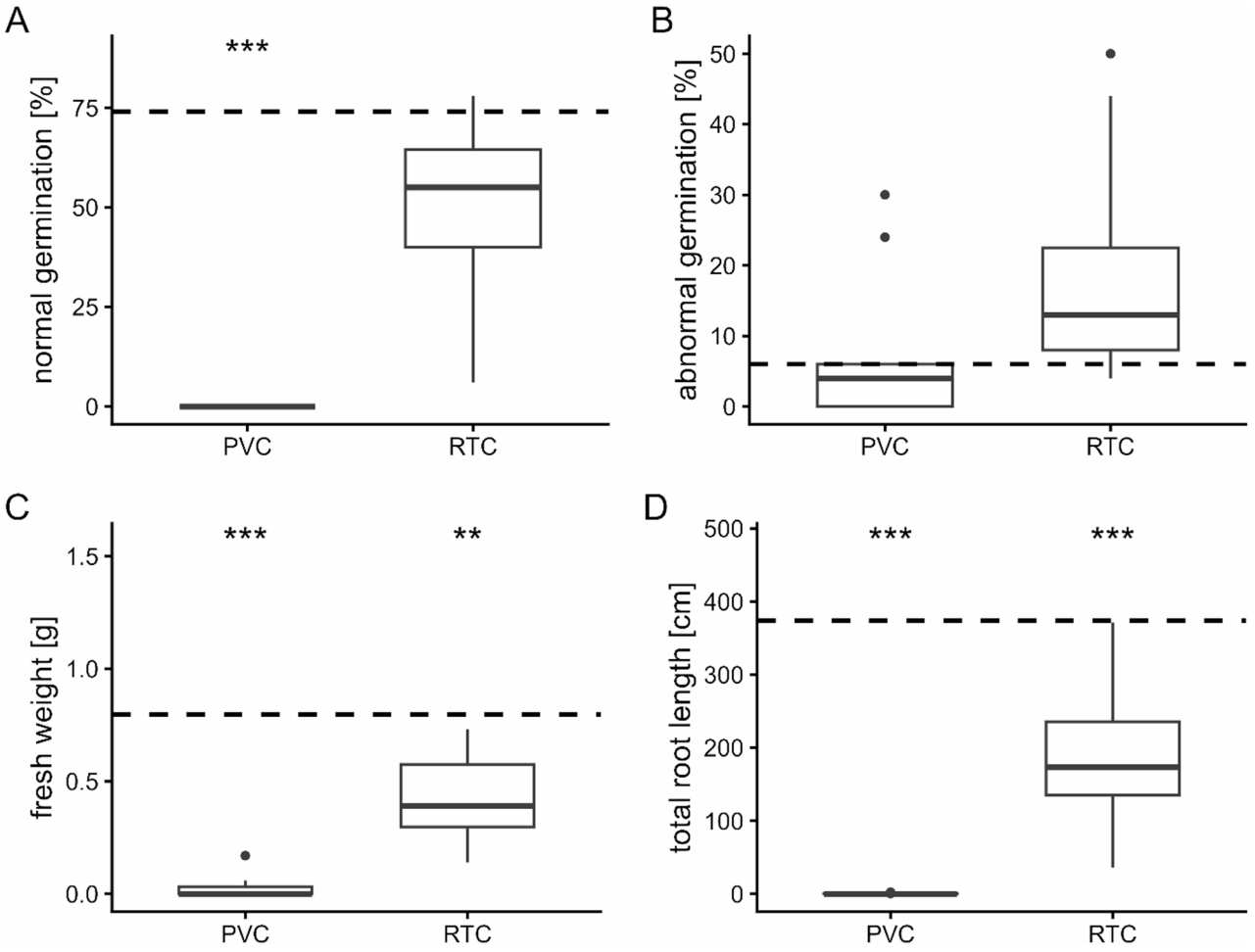


Fig. 1 Normal **(A)** and abnormal **(B)** germination rates, total fresh weight **(C)** and total root length **(D)** of *Lolium multiflorum* after 13 days of exposure to MP fragments (PVC or RTC) in a laboratory germination experiment. Data for PVC and RTC are pooled here across all fragment concentrations (0.5–2.0 g per Petri dish), and RTC data additionally pooled across size fractions (W1–W3). The dashed line indicates the mean of the controls. Asterisks indicate significances relative to the control based on Dunn's post-hoc tests with Bonferroni adjustment that followed Kruskal–Wallis (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

capable of inducing oxidative stress in plant tissues concurring with growth imbalance.

For RTC, there are considerably less exposure studies that investigated root growth parameters of crops or grassland species. Leifheit et al.. recorded root growth reductions [21], whereas Kim et al. did not detect any significant effects of RTC on root length of mung bean [22]. However, the focus of previous RTC research was mainly on soil mechanisms and in case of observed growth inhibitions, these were often attributed to Zn toxicity. Especially roots may be particularly vulnerable to elevated concentrations of Zn, as heavy metals often accumulate in root tissues, which may reduce toxicity in aboveground biomass [21].

The differences we observed between RTC and PVC effects may be related to different surface properties and the chemical composition (metal and additive content) of the chosen polymers. The PVC used was a pristine material, whereas RTC fragments represented recycled tire material. Due to the recycling process, surface and edges of RTC were sharp when compared to fine PVC fragment powder that was applied here. The aggravated effect of sharp-edged MP in comparison to rounded fragments has been shown in previous experiments, e.g. on duckweed [66]. Furthermore, the content of additives and heavy metals in RTC (selenium, sulphur, lead, cadmium and Zn) is usually larger than in PVC and has previously been reported to have inhibitory to phytotoxic effects on the growth of leguminous plants such as peas [67]. However, both these factors should have led to larger inhibitory effects of RTC compared to PVC, which was not observed here. The polymer-specific properties are addressed in more detail in the subsequent size and concentration comparison of PVC and RTC fragments.

Hypothesis B: Type and concentration effects in fragment treatments

To test how MP type and concentration potentially determine growth impairment, we analysed how germination and biomass responses differed within MP fragment treatments (PVC and RTC size fractions W1 – W3) across concentrations (0.5–2.0 g). The control group is displayed as a reference in the figures (dashed line) but excluded from the factorial design due to the absence of concentration levels.

Germination (normal and abnormal)

MP type was the dominant factor across the investigated parameters: two-way ANOVAs (type × concentration) revealed significant effects of MP type on normal germination, abnormal germination, fresh weight and root length; (all $p \leq 1.1 \times 10^{-5}$) (Fig. 2A-D). Post-hoc comparisons revealed that especially PVC had strong effects, as the contrasts between PVC and RTC treatments across the concentrations were frequently significant (emmeans, Bonferroni-adjusted, Table S1). To avoid conflation of MP type effects with RTC size effects, the contrasts among RTC size fractions (W1-W3) are separately reported in Table S3. For the higher concentrations of 1.5 and 2.0 g PVC, no biomass was generated (fresh weight = 0; total root length = 0; Fig. 2 + 3). This clearly indicates a dose effect that contributes to the detected differences between PVC and RTC.

The effects of MP concentration were endpoint-specific. Overall, PVC generally caused stronger impairment than RTC and differences among RTC sizes were not consistently significant across concentrations. Normal

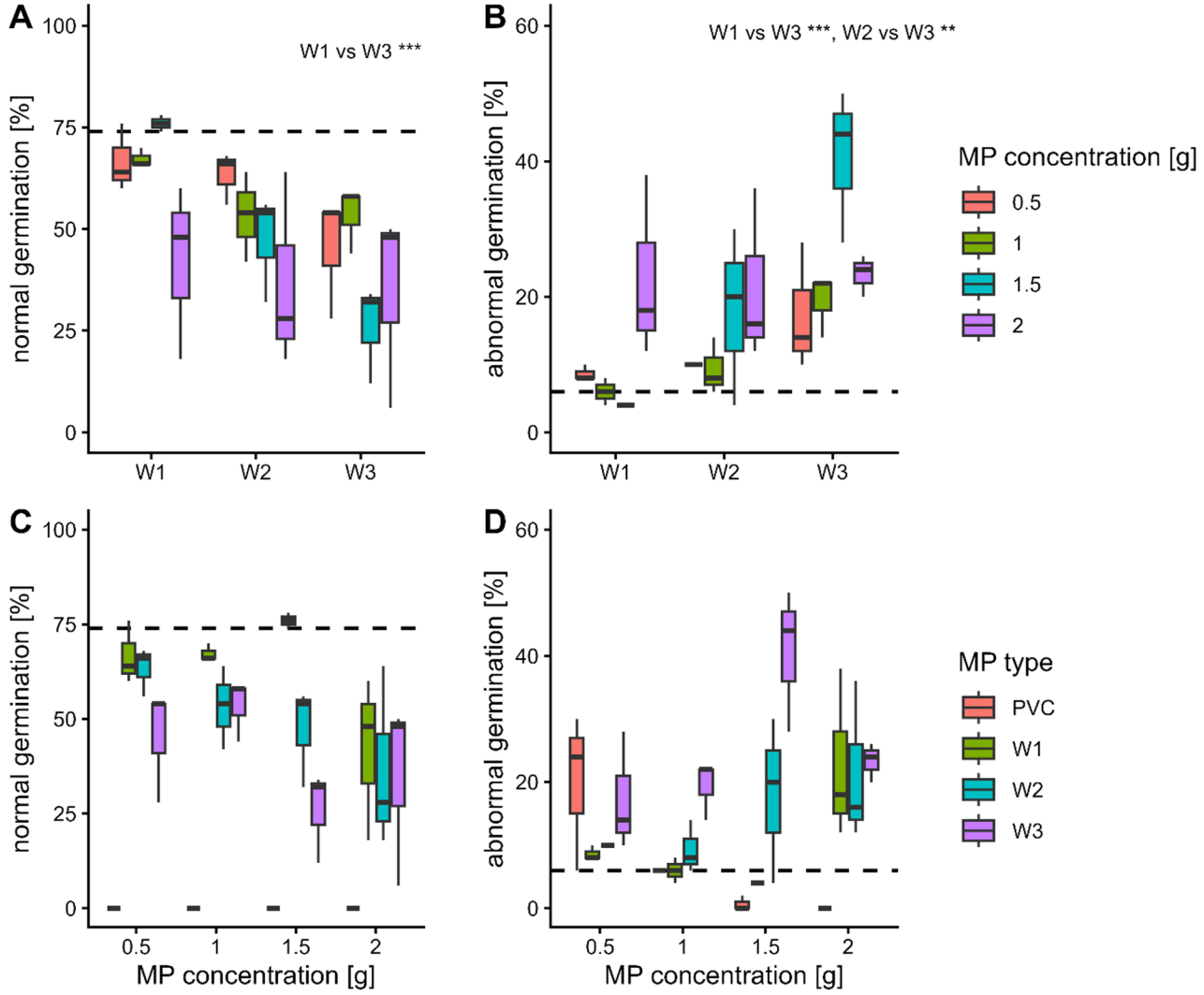


Fig. 2 Effects of MP fragment treatments on germination responses of *L. multiflorum*. **(A)** Normal and **(B)** abnormal germination for RTC size fractions W1-W3 across MP concentrations (0.5–2.0 g per Petri dish). **(C)** Normal and abnormal **(D)** germination across MP types (PVC and RTC size fractions) and MP concentrations. The dashed line indicates the mean of the control group. Asterisks denote significant differences among RTC size fractions (at 1.5 g). The PVC-RTC pairwise contrasts are shown in Table S1

germination ($p = 0.016$) and root length ($p < 0.001$) were significantly affected by MP concentration (normal germination: Fig. 2A, C); however, no significant concentration effects were recorded for abnormal germination ($p = 0.178$) and fresh weight ($p = 0.222$) (abnormal germination: Fig. 2B, D).

For the interaction of MP type and concentration, significance was only detected for abnormal germination ($p < 0.001$), but not for normal germination ($p = 0.125$) or fresh weight ($p = 0.872$); the interaction was close to significance for root length ($p = 0.060$). Among RTC size fractions (W1–W3), concentration-dependent effects were rare, significant differences only occurred at 1.5 g (emmeans, Bonferroni-adjusted; Table S3) (Fig. 2A-B). At this concentration, normal germination was higher in W1 than W3 treatments ($p_adj < 0.001$), abnormal germination was higher in W3 than W1 ($p_adj < 0.001$) and W2 treatments ($p_adj < 0.001$).

Additionally, concentration-specific Kruskal–Wallis tests followed by Dunn post-hoc tests (Bonferroni) were applied as a conservative robustness check, which resulted in fewer significances for pairwise contrasts. However, these tests confirmed the PVC–W3 difference in abnormal germination at 1.5 g ($p_adj = 0.016$) and several contrasts of PVC–W1 for normal germination, fresh weight and total root length at selected concentrations. Effect size estimates (η^2) indicated that MP type explained most of the variance across endpoint-specific ANOVAs (η^2 = 0.61–0.74), while MP concentration effects were smaller (η^2 = 0.04–0.10) and the type × concentration interaction was strongest for abnormal germination (η^2 = 0.35; Table S2).

Taken together, these findings partially support Hypothesis B. Concentration effects were endpoint-specific, and the effects of RTC size fractions were generally rare and did not show a consistent pattern of smallest fragments inducing the largest effects. MP type was the main driver of growth impairment, the strongest effects were observed for PVC, whereas concentration effects were not consistent across the growth responses. Importantly, the response to MP concentration may be non-linear: for PVC treatments, normal germination was already reduced at the lowest concentration ranges, indicating a threshold-like pattern across the tested concentration range rather than a gradual dose-response. For RTC, the clearest concentration-related effect occurred at 1.5 g (W3). Although oxidative stress markers or phytohormones were not quantified here, elevated abnormal germination may be consistent with stress-related seedling development and should be measured in future studies.

Germination impairment caused by different MP sizes has also been reported by Bosker et al., who exposed cress seeds to polystyrene MP on filter paper in petri dishes, the same setup that was used in this experiment [40]. In our experiment, PVC was applied in one fragment size and had a clearly stronger effect in comparison to all sizes of RTC. This result is in line with Esterhuizen et al. who also revealed PVC to cause the highest inhibition of germination in lotus among the tested polymers [33].

The apparent "size-related pattern" in normal germination reflects the differences between polymer types (PVC vs. RTC) rather than consistent differences among RTC types, where size-dependent effects were rare, except the pronounced effect on abnormal germination at 1.5 g W3. This indicates that the chosen concentration-range and spacing may have been too large (PVC) or too small (RTC) and may therefore have limited the ability to determine the lethal dose of these MPs for ryegrass.

Smaller MPs are generally assumed to induce more toxic effects in plants when compared to larger fragments [68]. We could not confirm this effect here in the chosen experimental setting. Although there was an overall negative influence of fragment addition, it could not be linked to specific particle sizes.

Fresh weight

There was a strong effect of MP type on fresh weight outcome ($p < 0.001$), whereas neither MP concentration ($p = 0.222$) nor their interaction ($p = 0.872$) was significant here, indicating that the degree of impairment was rather driven by type differences than consistent concentration-effects. Post-hoc tests (emmeans, Bonferroni-adjusted) revealed that PVC treatments frequently lowered fresh weight compared with RTC treatments across concentrations (Table S1). We could not confirm the hypothesis of size being negatively correlated to the effect size for fresh weight: within W1-W3, differences in fresh weight were not consistently significant and did therefore not follow the "small fragment – increasing effect pattern".

Fresh weight as a response parameter of plant growth under MP exposure has been studied with varying results. Colzi et al. found PVC and polypropylene to be those MPs causing the largest reductions in fresh (-35%) and dry weight of *Cucurbita pepo* L [61]. In a study on garden cress by Pignattelli et al., PVC also led to the greatest decrease in shoot weight among other polymers [69]. Previous results like these indicate that specific plant responses are dependent on MP type and some polymers such as PVC are more toxic than others [26]. However, there are considerably less studies regarding the effects of RTC fragments on plant growth parameters. Leifheit et al. tested the effect of RTC particles on growth parameters of *Allium porrum* and revealed that plant biomass was directly affected even at the smallest concentrations of RTC particles [21]. This is consistent with our experimental outcome, where a decrease in fresh biomass was observed across RTC treatments, but

this effect was not as strong as the effects of PVC in the same concentrations.

Total root length

MP type ($p < 0.001$) and concentration ($p < 0.001$) significantly affected total root length, whereas the type × concentration interaction was close to significance ($p = 0.060$). Post-hoc comparisons indicated that PVC frequently lowered total root lengths in comparison to RTC treatments across concentrations (Table S1). As stated above, PVC treatments of 1.5 g and 2 g resulted in no root biomass (total root length = 0 cm), indicating an abrupt growth inhibition for higher PVC concentrations (Fig. 3).

We chose total root length for analysis, since root parameters such as root activity, fresh and dry root biomass and root elongation are ranked on second position amongst plant parts that are directly or indirectly affected by MP [70]. As demonstrated here, total root length of *L. multiflorum* was strongly impacted by PVC treatments and impacted by RTC treatments, while concentration effects depended on treatment and endpoint. Adhesion of MP on root or seed surfaces can limit the flow of water and nutrients [71]. Root adhesion and entanglement with fragments may therefore be a very probable physical cause for that kind of root impairment [53]. This mechanism would also block the formation of further lateral roots.

As many reported MP-induced effects are concentration-dependent [33, 61], direct toxicity at high MP loads may also contribute to root growth impairment. Nei et al. showed that higher MP concentrations can elicit plant-specific growth response, but no phytotoxic reactions were observed in this experiment [72]. Colzi et al. likewise reported dose-dependent effects of PVC on plant growth responses of *Curcubita pepo L.*, especially in elemental profiles of root tissues.

In our study, RTC size-fraction effects were limited for germination and fresh weight, but were more apparent in total root length, highlighting that response parameters may react differently. Particle size may influence physical blockage, and smaller particles provide larger surface areas, that could enhance the amount of leachable compounds, potentially resulting in increased chemical

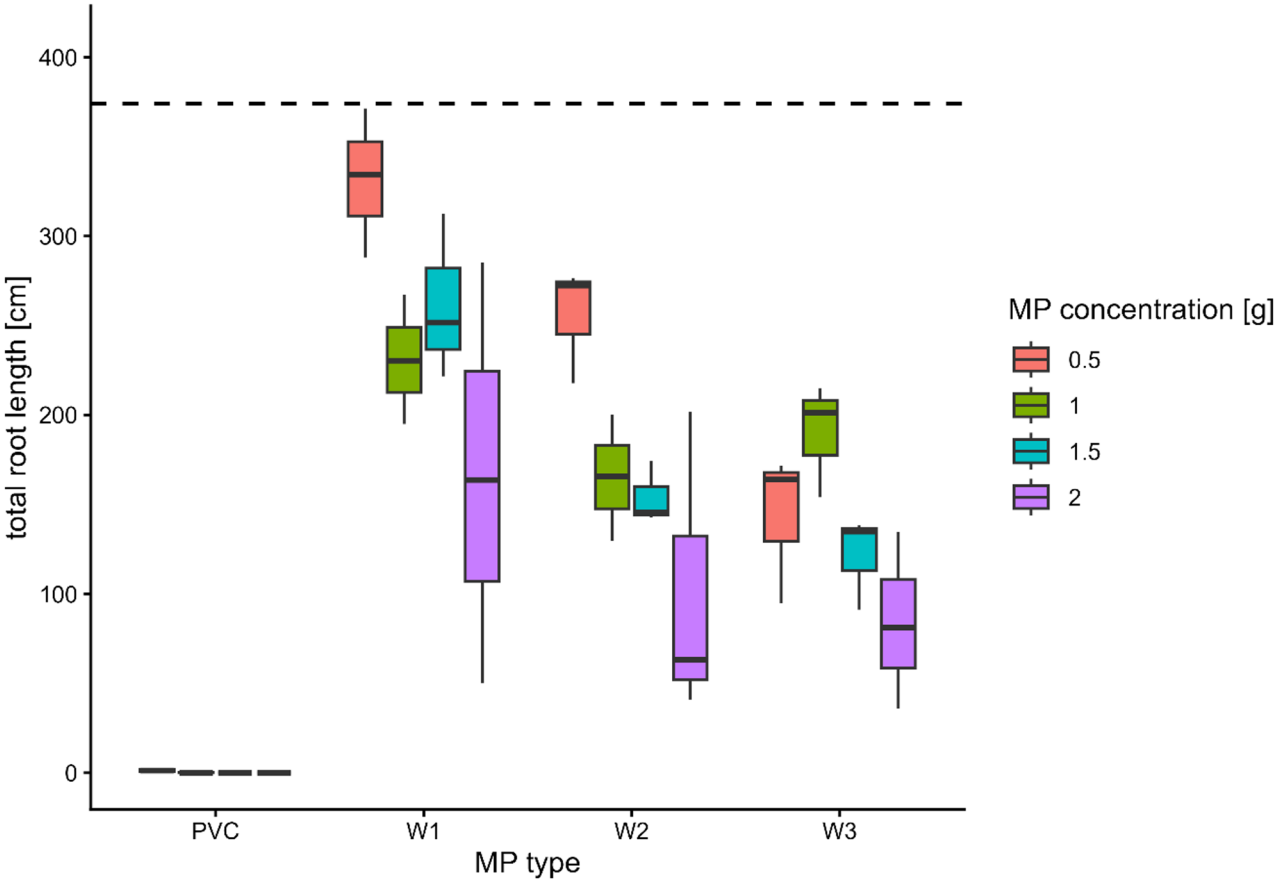


Fig. 3 Total root length [cm] of *L. multiflorum* after 13 days of exposure to microplastic (MP) fragments (PVC and RTC size fractions W1–W3) across MP concentrations (0.5–2.0 g per Petri dish). The boxes show distributions across replicates; the dashed horizontal line indicates the mean of the control group. Pairwise post-hoc comparisons are reported in Table S1 (PVC vs. W1-W3) and S3 (RTC size fractions W1-W3 only)

effects. Therefore, we studied leachate effects separately. As stated above, RTC was used as a proxy for TWP, differing from environmentally emitted TWP in particle composition and properties. This should be considered when results are extrapolated to field conditions.

Hypothesis C: Effects of MP leachates

Leachates were prepared as a single extract ratio; therefore, no interactions of MP type × concentration could be assessed for the leachate treatments.

The one-way ANOVA revealed a significant effect of MP type on the proportion of normal germination ($F(4, 10) = 35.53$, $p < 0.001$); this effect size was large ($\eta^2 = 0.93$, 95% CI [0.83, 1.00]). Tukey-adjusted post-hoc comparisons revealed that PVC leachates significantly reduced the proportion of normal germination compared to all other treatments (Fig. 4A): versus the control (mean difference = -0.74, $p_adj < 0.001$), versus W1 (mean difference = -0.69, $p_adj < 0.001$), versus W2 (mean difference = -0.61, $p_adj < 0.001$) and versus W3 (mean difference = -0.66, $p_adj < 0.001$). For RTC leachates, we did not observe any significant effects on the proportion of normal germination (all comparisons vs. control were not significant).

The one-way ANOVA revealed that abnormal germination was also strongly affected by MP leachates ($F(4, 10) = 20.86$, $p < 0.001$). The effect was very large ($\eta^2 = 0.89$, 95% CI [0.72, 1.00]). Tukey-adjusted post-hoc comparisons revealed that PVC induced significantly more abnormally germinated seeds in comparison to the control samples (Fig. 4B) (mean difference = 0.353, $p_adj = < 0.001$), to W1 (mean difference = 0.327, $p_adj < 0.001$), to W2 (mean difference = 0.300, $p_adj < 0.001$) and also to W3 (mean difference = 0.347, $p_adj < 0.001$). There were no significances detectable between RTC leachates and the control (all $p_adj > 0.78$). The results on normal and abnormal germination caused by leachates are qualitatively similar to the results of the lowest concentration (0.5 g) of MP fragments. For PVC, we noticed higher abnormal germination under leachate treatments than under fragment exposure (descriptive comparison).

This pattern is generally consistent with leachate-mediated effects. However, because leachates were prepared at a single extraction ratio and not chemically characterized, the drivers of the PVC leachate response cannot be disentangled here, this effect may also be concentration-dependent [73]. Results should therefore be interpreted cautiously. This may help to explain why PVC leachates elicited stronger responses than RTC leachates under the applied extraction conditions. Although RTC commonly contains large amounts of metals and specific tire-additives, stronger responses were observed for PVC leachates in our study, indicating that differences in composition and bioavailability of the leachate compounds may have had a stronger influence than the total additive load. Depending on the processing, PVC products may contain additives with leaching potential such as plasticizers and stabilizers, including phthalates and organotins. Differences in leaching kinetics may therefore

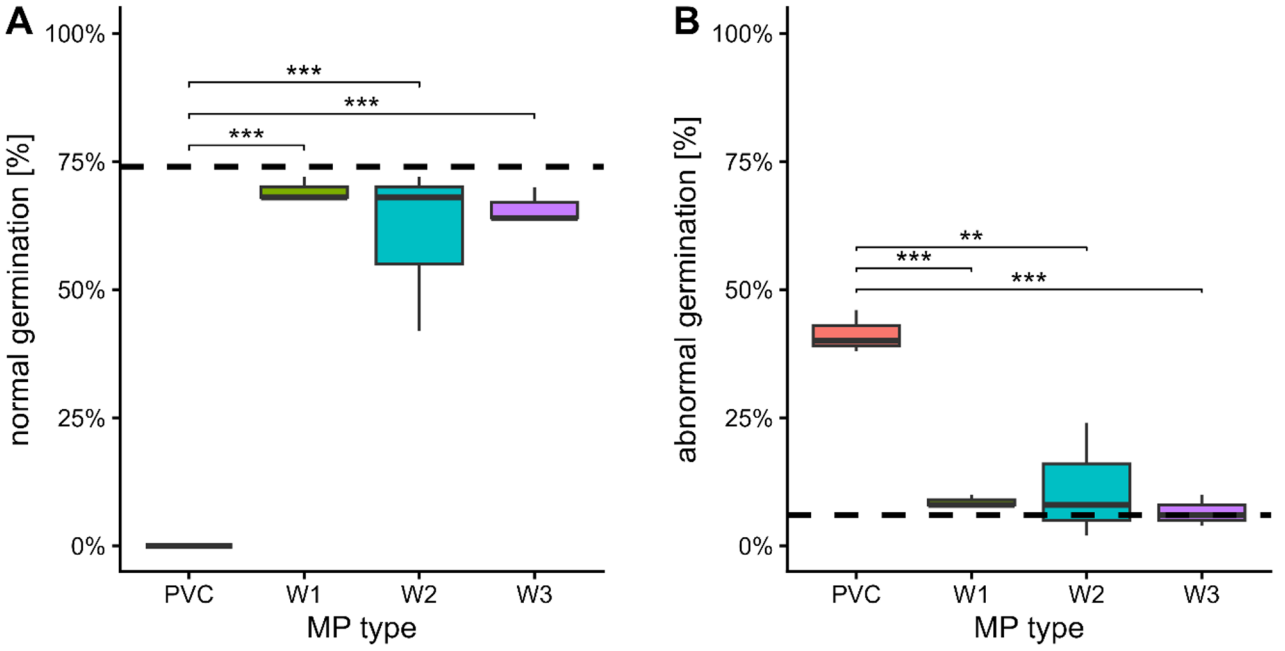


Fig. 4 Normal **(A)** and abnormal **(B)** germination of *L. multiflorum* after 13 days exposure to MP (microplastic) leachates (PVC and RTC leachates W1-W3; $n = 3$ per treatment). The dashed line indicates the mean of the control group. Brackets indicate significant pairwise comparisons of leachate treatments based on Tukey-adjusted post-hoc tests (* p_adj < 0.05, ** p_adj < 0.01, *** p_adj < 0.001)

determine the contrasting response patterns. However, as PVC and RTC leachates were not further characterized, the causal relationships cannot be identified here in detail.

As stated above, the proportion of abnormal germination was also large for PVC fragments, indicating even higher toxicity of these particles. While PVC leachate exposure had strong effects on germination, this pattern was not consistent for RTC leachates. This resulted in a low number of viable seedlings, due to a severely affected normal germination rate and a parallel increase of abnormally germinated seeds with consequences for other growth patterns.

Fresh biomass ranged from 0.22 ± 0.08 g (PVC) to 0.80 ± 0.57 g (control) and there were no significant effects of any leachate treatment on fresh weight (χ^2 = 6.95, df = 4, p = 0.138). Compared to MP fragments, fresh weight under leachate treatments tended to be higher than under fragment exposure (descriptive comparison). However, we did not detect significances among the leachate treatments in comparison to the controls, the results should therefore be interpreted with caution. Larger fresh weights observed for leachate treatments were not consistently accompanied by root elongation in comparison to fragment treatments:

The one-way ANOVA on total root length revealed a significant effect of MP type, $F(4, 10) = 20.39$, $p < 0.001$. The effect size was large (η^2 = 0.89, 95% CI [0.71, 1.00]). Root lengths ranged from 9.82 ± 5.57 cm for PVC leachates to 374.0 ± 66.68 cm for the control samples. Tukey-adjusted post-hoc comparisons revealed significantly shorter root lengths for PVC leachates compared with the control (mean difference [cm] = -364.19, $p_adj < 0.001$), and also shorter root lengths when compared to W1 (mean difference = -301.62, $p_adj < 0.001$), W2 (mean difference = -197.61, $p_adj < 0.001$) and W3 (mean difference = -258.34, $p_adj < 0.001$). Among RTC leachates, W2 generated shorter root lengths when compared to the control (mean difference = -166.58 cm, $p_adj = 0.022$), whereas there were no significant differences between W1 or W3 and the controls. When compared to fragment treatments, we observed longer roots for PVC and W3 leachates and shorter root lengths for W1 and W2 leachates (descriptive comparison).

Most of the research conducted on PVC leachates so far has focused on aquatic organisms that were clearly negatively affected by PVC leachates [70, 73]. Currently, there is insufficient information published on the effects of MP leachates on plants, and in particular on the effects of PVC leachates on grassland species. However, Wang et al. [65] revealed root reductions of wheat caused by photoaged PVC leachates. In their experiment, the authors determined leachate concentration and type of additives to be major drivers for the observed plant growth impairment. In our experiment, one concentration ratio was applied for the preparation of MP leachates and chemical composition of additives was not further analysed. Therefore, we are unable to confirm whether differences in leachates composition of PVC and RTC explain the differences in plant responses to leachate and fragment treatments.

Contrary to our original hypothesis, RTC leachates did not elicit stronger effects on the selected growth parameters than PVC leachates. However, our results for RTC leachates are consistent with Castan et al., who revealed no toxic effects of RTC leachates on lettuce [24]. RTC effects are generally discussed with emphasis on their chemical composition, including heavy metals such as Zn, originating from ZnO vulcanization process. Although original metal content is decreased throughout the tire recycling process, Zn still is a major component of RTC with concentrations 5-500 folds higher than in other MPs. The bioavailability of Zn (and other tire-derived compounds) depends on material characteristics, aging, reprocessing and exposure conditions [54]. Ryegrass species are reported to react sensitively to elevated metal concentrations in the seed surrounding [71]. Zn can accumulate in below-ground plant tissues; therefore, metal-release into moist germination environment remains a plausible, but unconfirmed contributor to RTC leachate effects. However, since we did not quantify Zn in the respective leachates, the contribution of Zn to the observed effects cannot fully be determined here. Capolupo et al. [74] reported strong effects of both RTC and PVC leachates in aquatic settings, highlighting that both materials are highly diverse and may potentially release compounds that are biologically relevant. This study however addressed aquatic species and is therefore not fully comparable to our results.

Taken together, our findings partially support Hypothesis C: exposure to MP leachates predominantly affected germination patterns and root elongation, whereas fragment exposure may additionally cause reductions in germination, root length and biomass generation potentially, involving physical interference. However, addressing the underlying mechanisms was beyond the scope of this study.

Hypothesis D: MP-related shifts in δ^{13}C-signatures

δ^{13}C-signatures of shoots were analysed to assess MP-related differences in early physiological processes. For MP fragments, the two-way ANOVA (MP type × MP concentration) revealed that MP concentration significantly affected δ^{13}C-signatures ($F(3,27) = 4.30$, $p = 0.013$), whereas there were no significant effects of MP type, $F(3,27) = 2.47$, $p = 0.083$) and the interaction ($F(7,27) = 1.66$, $p = 0.161$) (Fig. 5A). Bonferroni-adjusted post-hoc comparisons did not reveal any consistent

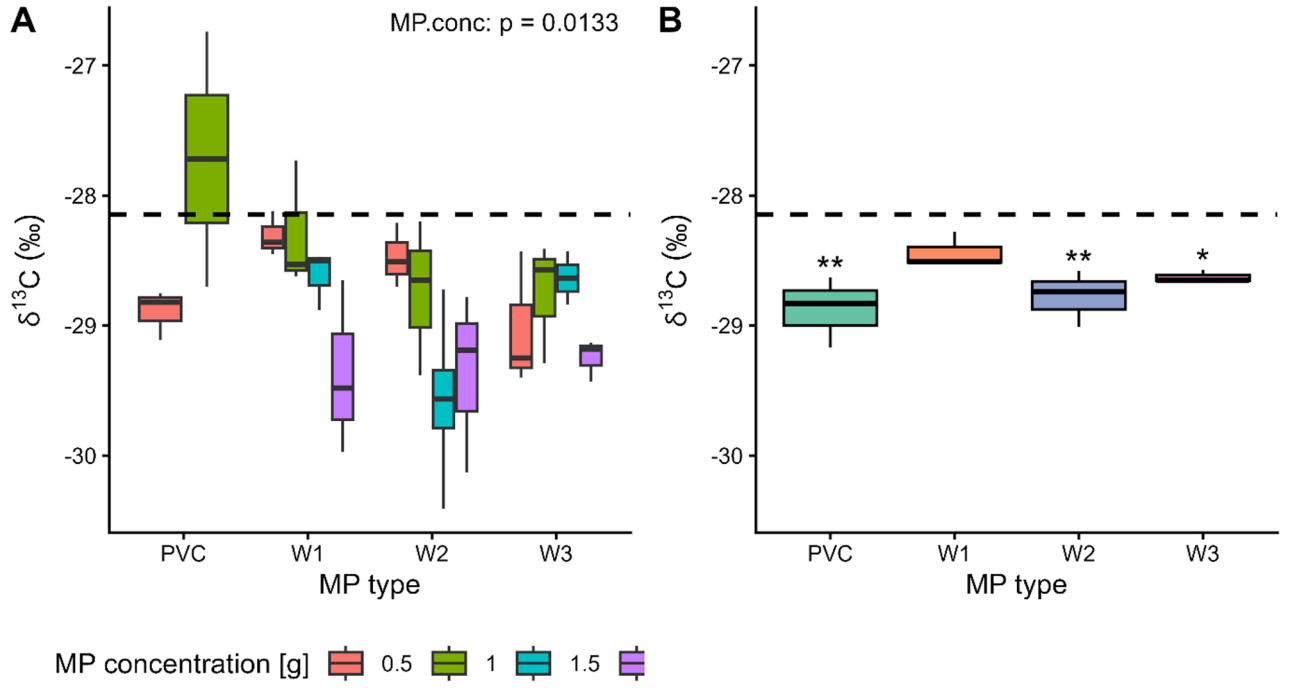


Fig. 5 δ^{13}C-signatures (‰) of *L. multiflorum* shoots after 13 days of exposure to microplastic (MP) fragments and leachates in a laboratory germination trial. **(A)** MP fragment treatments (PVC; RTC size fractions W1-W3) across MP concentrations (0.5; 1; 1.5; 2; $n = 3$ per treatment), colours indicate MP concentrations. **(B)** MP leachate treatments (PVC, RTC size fractions W1-W3, $n = 3$ per treatment). Colours indicate MP type and the dashed line indicates the mean of the control group. Asterisks indicate significant differences of treatments versus the control (Tukey-adjusted: * p_adj < 0.05, ** p_adj < 0.01)

differences among RTC size fractions across the concentrations ($p > 0.088$).

For leachates, the one-way ANOVA revealed a significant effect of MP type on the δ^{13}C-signatures $F(4, 10) = 8.66$, $p = 0.003$). This effect size was large ($\eta^2 = 0.78$, 95% CI [0.41, 1.00]). Post-hoc tests revealed that PVC (mean difference = -0.73, $p = 0.003$), W2 (mean difference = -0.63, $p = 0.008$) and W3 (mean difference = -0.48, $p = 0.040$) were significantly more depleted than the control samples, which is consistent with alterations in stomatal regulation, although we did not measure these mechanisms here. None of the MP leachate treatments was significantly enriched but instead even more depleted than the control samples (Fig. 5B).

Across the fragment treatments, shoot δ^{13}C signatures did not correlate with fresh biomass (Spearman's $\rho = 0.11$, $p = 0.490$), but were positively correlated with total root length ($\rho = 0.47$, $p = 0.002$). For leachate treatments, δ^{13}C signatures were positively correlated with fresh biomass ($\rho = 0.66$, $p = 0.024$) and with total root length ($\rho = 0.72$, $p = 0.011$).

The pure signatures of the tested MP fragments were as follows: PVC: -31.43‰; W1: -26.68‰; W2: -27.15‰; W3: -27.39‰. Generally, if carbon derived from polymers contributed to plant tissue δ^{13}C-signatures, this may have led to a shift towards depletion for PVC and enrichment for RTC, when compared to the controls. However, we did not detect such patterns, indicating that the isotopic shifts were more likely consistent with physiological responses (water-use/ stomatal regulation) rather than by direct incorporation of polymer-derived carbon.

In principle, depletion of δ^{13}C in C3 plants is consistent with increased carbon isotope discrimination and may reflect changes in stomatal conductance and/or photosynthetic assimilation. Thus, the depletion observed throughout the data may indicate either higher stomatal conductance (e.g. due to more water availability) or less reduced assimilation under MP exposure, resulting in higher intercellular CO_2 concentrations. Because water availability is supposed to be impaired rather than improved by MP exposure, reduced assimilation might have been the cause of the depletion, in line with reduced biomass observed in some treatments. However, stomatal conductance and photosynthetic performance were not directly measured in our study, the underlying mechanisms therefore remain undetected here.

Within MP treatments (fragments and leachates), δ^{13}C was more consistently associated with total root length than with fresh biomass, indicating that the observed isotopic shifts may co-occur with growth responses. However, because data were pooled across MP types (and, for fragment treatments concentrations), these correlations cannot be attributed to specific treatments.

This provides additional context for the observed growth responses across fragment and leachate treatments. As shown above, fragment exposure did not elicit

consistent size-related effects, except for effects on root elongation. At the same time, the effect of fragments on growth parameters was often larger than that of leachates, indicating an added (physical) effect of fragments over leachates.

MP and drought-induced water stress may be closely linked as both can act in concert and cause similar inhibitions in plant performance [75]. Wu et al. showed that PVC MPs can externally block the stomata of *Brassica napa* and reduce fresh weight, but not dry weight, suggesting that PVC may affect plant water relations in addition to the impairment of biomass production [76, 77]. In our study, MP fragments reduced root growth, yet we did not observe consistent parallel effects on $\delta^{13}C$. Because fresh weight was not reduced in all fragment treatments, reduced water demand is unlikely to have masked a water availability effect here. One possible explanation is that MP fragments increased water-holding capacity, thus making more water available to the plants. This was also suggested by Bakhshaee et al. [78]. However, leachates affected $\delta^{13}C$ more strongly than fragments, indicating that leachable compounds may have contributed to the observed shifts and growth responses. In direct comparison of leachate and fragment treatments, we observed plants with PVC and W3 leachates being more depleted than W1 and W2. Interestingly, observed root lengths were also enlarged for the same leachate treatments. The action-mode of MP displayed in $\delta^{13}C$-signatures may therefore be a combination of both physical and chemical effects.

Roots tips and root hairs are known to be critical for physical MP blockage that can affect nutrient uptake [79]. If plants were hindered in root hair growth performance, we should have seen enrichment throughout the data. It may therefore also be possible that MP fragments hinder root elongation but have no effect on the formation of root hairs.

Ryegrass may be capable of enlarging uptake pathways for nutrients and water via changes in root architecture: The increased formation of lateral roots and the associated further branching of the root network may satisfy proper flow of nutrients and water but was not analysed here as we concentrated on root length. Since the root system determines the growth of above-ground biomass, and an adequate leaf development and photosynthesis rate are essential for proper plant establishment [80], alterations in root branching may still allow plants affected by MP to fulfil the nutritional demands of juvenile plant development. Species with a rapid juvenile development such as grasses [81] may be particularly capable of compensating such temporarily limited access to resources.

Stimulation of lateral root growth of *Oryza sativa* as a consequence to MP exposure was observed by Wu et al. [42], who stated that phytotoxicity of larger MPs may be even stronger than the one of smaller particles. Their results are in line with those published by Zhou et al., who also observed depressions of primary roots and root mass but concomitantly increases of lateral root mass of *Oryza sativa* after exposure to MP [82]. Li et al. demonstrated specifically these lateral roots as alternate crack-entry pathways for small MP [83]. Enlargement of the lateral root architecture may therefore also expand the uptake pathways of ryegrass and lead to more potential uptake routes for plants growing with MP presence than without.

Although these studies used different model plants, MP types and sizes, their results are still in line with ours, as we observed significant decreases in total root length, but did not further differentiate between root types. The reduction in primary root length may outweigh any stimulation of lateral root growth, resulting in overall decrease in total root length, as observed here. Such compensatory shifts towards more lateral roots could still increase the total number of potential uptake roots in plants exposed to MPs, despite reduced total root length.

Conclusion

This study analysed the effects of PVC and RTC MPs in the form of fragments or leachates on normal and abnormal germination rates, total root length, fresh weight and $\delta^{13}C$-signatures of *L. multiflorum* in a laboratory germination experiment. MP fragments, depending on their original polymer type and concentration, exerted negative effects on germination and the associated growth parameters of *L. multiflorum*. The proportion of abnormally germinated seeds as a parameter of plant establishment and viability has so far not been considered in MP exposure research but stands out among our selected parameters. As germination success is a key factor for plant establishment, an increased number of deformed seedlings is likely to cause reductions in active plant material.

The impact of MP fragment size as an inhibitory factor was confirmed for root length but not for all plant growth parameters in our experiment. Nevertheless, PVC fragments showed concentration-dependent inhibitory effects, especially for germination and root length. The concentration effects for RTC size fractions were not consistent and were endpoint-specific. MP leachates also negatively influenced growth responses: Fresh weight was not significantly affected by leachate treatments, whereas the responses of germination and total root length were reduced, particularly for PVC leachates. Both PVC fragments and leachates caused more inhibitory effects on growth than RTC treatments did.

Interestingly, the $\delta^{13}C$-signatures did not show a shift consistent with reduced water status under MP

exposure; as MP treatments tended be more depleted than enriched. Because $\delta^{13}C$ may reflect both, stomatal regulation and photosynthetic processes, these shifts cannot be considered to reflect water availability alone. Follow-up studies should also include physiological analyses on photosynthetic performance.

We chose MP concentrations ranging from 0.5 to 2.0 g per Petri dish, to measure effects under controlled conditions and to compare MP types and RTC size fractions. These concentrations are likely higher than reported MP loads in agricultural soils; therefore, our results should be interpreted in terms of hazard potential rather than direct field occurrences.

Our findings highlight that chemical effects caused by MPs play an important role in germination and plant growth, increasing their impact beyond the directly touched and physically impaired surfaces. This was not only the case for RTC, which is known to come with a suite of added chemicals, but even stronger so for PVC. The mode of action of MP seems therefore to be based on a combination of both chemical and physical effects.

Abbreviations

PVC	Polyvinylchloride
TWP	Tire Wear Particles
Zn	Zinc
MP	Microplastic
RTC	Recycled Tire Crumb
EA	Elemental Analyser
IRMS	Isotope Ratio Mass Spectrometer
IAEA	International Atomic Energy Agency
VPDB	Vienna Peedee belemnite
ANOVA	Analysis of Variance
SEM	Standard Error of Mean

Supplementary Information

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Supplementary Material 1

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Author contributions
H.C.C. and N.W.-M. did the conceptualisation and H.C. wrote the manuscript. N.W.-M. supervised and extensively reviewed formal analysis, writing and editing. J.M. extensively reviewed statistics and writing.

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Data availability
The datasets that were generated and analysed for the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval
Not applicable. This study did not involve human participants, personal data, property, or animals.

Competing interests
The authors declare no competing interests.

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