

Abstract

Biological invasions have major impacts on a variety of ecosystems and threaten native biodiversity. Earthworms have been absent from northern parts of North America since the last ice age, but non-native earthworms were recently introduced there and are now being spread by human activities. While past work has shown that plant communities in earthworm-invaded areas change towards a lower diversity mainly dominated by grasses, the underlying mechanisms related to changes in the biotic interactions of the plants are not well understood. Here, we used a trait-based approach to study the effect of earthworms on interspecific plant competition and aboveground herbivory. We conducted a microcosm experiment in a growth chamber with a full-factorial design using three plant species native to northern North American deciduous forests, *Poa palustris* (grass), *Symphyotrichum laeve* (herb), and *Vicia americana* (legume), either growing in monoculture or in a mixture of three. These plant community treatments were crossed with earthworm (presence or absence) and herbivore (presence or absence) treatments. Eight out of the eleven above- and belowground plant functional traits studied were significantly affected by earthworms, either by a general effect or in interaction with plant species identity, plant diversity level, and/or herbivore. Earthworms increased the aboveground productivity and the number of inflorescences of the grass *P. palustris*. Further, earthworms countervailed the increasing effect of herbivores on root tissue density of all species, and earthworms and herbivores individually increased the average root diameter of *S. laeve* in monoculture, but decreased it in mixture. In this study, earthworm presence gave a competitive advantage to the grass species *P. palustris* by inducing changes in plant functional traits. Our results suggest that invasive earthworms can alter competitive and multitrophic interactions of plants, shedding light on some of the mechanisms behind invasive earthworm-induced plant community changes in northern North America forests.

Keywords: biological invasion, competition, detritivore, herbivory, plant functional traits

Introduction

Biological invasions occur globally and are among the most significant direct causes of native biodiversity loss worldwide (Díaz et al., 2019; Ehrenfeld, 2010; Murphy & Romanuk, 2014). Dramatic changes in biodiversity can be due to invasive species acting as ecosystem engineers, such as European earthworms in northern North America (Blouin et al., 2013; Bohlen et al., 2004a; Ehrenfeld, 2010; Ferlian et al., 2018). Much of the native flora and fauna was eradicated in this region during the last ice age, including earthworms (Gates, 1982), which have been reintroduced by European settlers within the last centuries (Hendrix & Bohlen, 2002).

In areas where invasive earthworms are abundant, they alter the physico-chemical properties of the soil (Bohlen et al., 2004a; Ferlian et al., 2020; Hale et al., 2005). The litter coverage is reduced, soil horizons partly homogenized, nutrients redistributed, and soil pH increased while soil water content decreased (Blouin et al., 2013; Eisenhauer et al., 2007; Ferlian et al., 2020; Hale et al., 2005). These modifications of abiotic conditions alter the habitat for soil-living organisms (Eisenhauer et al., 2007; McLean & Parkinson, 2000). As a consequence, the community composition of soil microbes and invertebrates is significantly altered, while soil fauna diversity and density decrease with earthworm invasion (Eisenhauer et al., 2007; Ferlian et al., 2018).

Further, earthworms can act as an ecological filter on seeds, seedlings, and plants (Eisenhauer & Scheu, 2008b; Forey et al., 2011; Frelich et al., 2012) impacting plant species establishment. The diversity of understory plants was reported to decrease, and the species and functional community composition change significantly in response to the invasion of earthworms (Bohlen et al., 2004b; Craven et al., 2017; Hale et al., 2006). However, it was also shown that there are winners and losers: while some grass and non-native plant species benefit from earthworm invasion (Craven et al., 2017), the richness and abundance of herbs (Hale et al., 2006) and legumes (Eisenhauer et al., 2007) decrease in invaded forests, leading in some cases to sparse vegetation with only few herbaceous plant species left (Bohlen et al., 2004b).

Nevertheless, the total plant cover was overall shown to increase and be dominated by graminoids (Craven et al., 2017), indicating that earthworms may change competitive interactions by benefitting certain plant species (Eisenhauer & Scheu, 2008a; Nuzzo et al., 2009; Wurst et al., 2005). In this context, our study aims to disentangle the mechanisms underlying plant community changes due to earthworm invasion, by focusing on biotic interactions and changes in plant functional traits (*i.e.* “morpho-physio-phenological traits which impact fitness indirectly via their effects on growth, reproduction and survival” (Violle et al., 2007)). Plant functional traits related to development and resource uptake can be affected by earthworm presence (Thouvenot et al., 2021). Previous studies mostly focusing on native plant and earthworm species have shown among others that earthworm activity enhanced shoot biomass (Groenigen et al., 2014; Scheu, 2003), plant growth (Poveda et al., 2005; Wurst et al., 2003; Xiao et al., 2018), seed quantity (Poveda et al., 2005), and fine-root growth (Blume-Werry et al., 2020), with some variation across plant species and functional groups.

The provision of available nitrogen is considered as the main pathway of earthworms to affect plants (van Groenigen et al., 2014). Earthworms enhance the nitrogen uptake of grasses, herbs, and legumes from organic litter and soil (Eisenhauer & Scheu, 2008a; Wurst et al., 2003). Furthermore, their effects on soil structure, microbiota, and water availability can also influence plant growth (Blouin et al., 2013; Cameron et al., 2014; Eisenhauer et al., 2012, Scheu, 2003), and thus earthworms might select for plant species that tolerate drought, can germinate on bare soil, and spread vegetatively (Eisenhauer et al., 2012). By having dissimilar effects on plant functional traits of different species, invasive earthworms can affect biotic interactions of plants, *e.g.* competition and herbivory (Blouin et al., 2013; Eisenhauer et al., 2009) and thus promote certain plant species. For example, species with a more flexible rooting strategy were able to better adapt to the presence of invasive earthworms and forage in their nutrient-rich burrows (Cameron et al., 2014). According to the plant economics spectrum (Reich, 2014), grasses are considered as fast-growing plants that efficiently exploit nutrient patches in contrast to slow growing herbs (Lavorel et al., 2007; Linder et al., 2018). Therefore, grasses competitiveness could be increased by earthworm invasion (Craven et al.,

2017), as grasses potentially build more root biomass and take up more nitrogen when earthworms are present (Eisenhauer & Scheu, 2008a; Wurst et al., 2005). Indeed, it was shown that legumes had less biomass when growing with grasses in the presence of earthworms (Eisenhauer & Scheu, 2008a), which indicates an elevated competitive disadvantage that might explain the lower legume cover and biomass in earthworm-invaded aspen forests (Eisenhauer et al., 2007).

In addition to plant competition, invasive earthworms may alter multitrophic interactions of plants. Earthworms can affect aboveground herbivory through plant-mediated effects (Wurst et al., 2003; Wurst, 2010; Xiao et al., 2018), positively (Newington et al., 2004; Thakur et al., 2020), or negatively (Zaller et al., 2013). Loranger et al. (2012) reported that the nutrient content and toughness of leaves are the most important plant traits linked to herbivory in grassland: high plant tissue nitrogen content increases while high leaves' toughness decreases leaves' palatability to herbivores. Leaf nitrogen content was shown to be enhanced by earthworm activity (Newington et al., 2004; Wurst et al., 2004, Xiao et al., 2015, Blume-Werry et al., 2020), while inconsistent effects were shown on leaf dry matter content, a trait that could be used as proxy for leaf toughness (Elger & Willby, 2003; Thakur et al., 2020; Thouvenot et al., 2021).

To improve the mechanistic understanding of recorded effects of invasive earthworms on native understory plant communities in northern North American forests (e.g. Craven et al., 2017), we set up a microcosm experiment in a growth chamber with plants belonging to three functional groups (grasses, herbs, and legumes), either in monoculture or mixture. Each plant community was subjected to the presence and absence of earthworms and aboveground herbivores, respectively, resulting in a full-factorial design. To shed light on potential treatment effects, we assessed a set of plant functional traits that are likely to play critical roles in plant resource use, competition, and responses to herbivores. As earthworms were expected to increase nutrient availability for plants, we hypothesized that (1) invasive earthworms increase overall productivity of native plant species. Given that grasses can exploit soil nutrients rapidly

(Linder et al., 2018) and their response to invasive earthworms is more pronounced than that of herbs (Thouvenot et al., 2021), we hypothesized that (2) grasses become stronger competitors and show increased growth, while herbs and legumes show decreased growth due to high competition from grasses in the presence of earthworms. Moreover, as herbivory can affect plant traits (Heinze, 2020), we expected (3) potential short-term effects of aboveground herbivory on plant traits (Poveda et al., 2003), while (4) invasive earthworms may modulate herbivory effects due to changes in soil nutrient availability (Eisenhauer & Scheu, 2008a). Further, we (5) expected that the effects of invasive earthworms and aboveground herbivory depend on plant community composition, e.g. with grasses benefiting most from the presence of earthworms and herbivores in plant mixtures (Eisenhauer & Scheu, 2008a).

Methods

Experimental set-up

Earthworm effects on biotic interactions (e.g. competition and herbivory) were studied in a microcosm experiment under controlled conditions using a full-factorial design with four types of plant communities and four earthworms/herbivore treatments. Three species of the native plant community from Canadian forest understories (Hallworth & Chinnappa, 1997; Royer & Dickinson, 2007), namely *Poa palustris* (grass, purchased at Sheffield's Seed Co. Inc, USA), *Vicia americana* (legume, purchased at ALCLA Native Plants, Canada), and *Symphyotrichum laeve* (herb, purchased at Wild About Flowers, Canada), were each grown in monoculture and in mixture (*i.e.* all three together); resulting in four plant community treatments. We manipulated the presence of earthworms (*Lumbricus terrestris*, anecic species from Europe, but invasive across northern North America; Hendrix et al., 2006, 2008) and aboveground herbivores (larvae of *Spodoptera exigua*, chewing generalists) in a full-factorial design: (1) control (no earthworms, no herbivores), (2) with earthworms only, (3) with herbivores only, and (4) with both earthworms and herbivores. Each of the 16 different treatment combinations (4x plant communities, 2x earthworms, 2x herbivores) was replicated 6 times, resulting in 96 microcosms. The experiment was conducted for 82 days. Microcosms were randomly

distributed on four tables in an air temperature-controlled climate chamber with 60% humidity, at a day/night cycle of 16/8h and $23/15 \pm 0.9^{\circ}\text{C}$ (mean \pm sd), with a light intensity of around 400-450 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation. Each microcosm was watered with 20-100 ml of water every 2-3 days during the experiment. Microcosms were randomly redistributed between the tables every two weeks, but always at least one replicate of each treatment was placed on each table.

Microcosms consisted of PVC tubes (inner diameter 10 cm, height 24 cm) with a 700 μm mesh glued to the bottom to allow drainage of water. At the top of the tube, a barrier of transparent plastic film (height 15 cm) was attached to prevent earthworms from escaping (Eisenhauer et al., 2012). Soil (sandy-clay soil, pH= 7.38; N= 0.06%; C= 1.20%; C:N= 20.98) was ordered from a commercial supplier (Kies Direkt, Germany), and each microcosm was filled with 3 kg of sterile (*i.e.* autoclaved at 120° ; 90 min) soil. To leach nutrients and compounds released during the sterilization procedure, 1 l of water was added to each microcosm.

Then, a soil microbial inoculation, using native soil that was sampled in September 2020 in an uninvaded Aspen forest of the Kananaskis Valley (Alberta, Canada), was performed by directly mixing soil and by using a microbial wash. Native soil was shipped frozen, stored at -20°C until usage, and, after defrosting, sieved through a 4 mm mesh. For each microcosm, we homogenized the 3 kg of sterilized soil with around 137 g of the Canadian soil in a sterilized (70% ethanol) bucket, before filling it back to the microcosm. To perform the microbial wash, we mixed 46 g of Canadian soil with 100 ml of water and poured it into each microcosm, followed by another 100 ml of water to dissolve the remains of the Canadian soil from the vessel. To ensure microbial community development, we stored the microcosms for ten days at room temperature ($20-25^{\circ}\text{C}$) and held their soil water content at 40-60% of their maximum water holding capacity.

Plant material

Seeds were sown in soil from the Bad Lauchstädt Experimental Research Station of the Helmholtz Centre for Environmental Research- UFZ ($11^{\circ}53'\text{E}$ $51^{\circ}24'\text{N}$). Prior to sowing, the

soil was autoclaved (120°C; 90 min), washed with tap water, and inoculated using both approaches described above. Seeds of *V. americana* were scarified using sandpaper (grit size 150) and sown at 3-6 mm soil depth, *S. laeve* at 2 mm soil depth, and *P. palustris* at the soil surface. Seeds then germinated in a reach-in climate chamber (night temperature: 18°C, day temperature: 22°C, duration day/night: 16 h/8 h; light intensity day: 450 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (partial light at 85%), humidity: 70%). After six weeks, the seedlings were transplanted into microcosms, and their vegetative height was measured (mean \pm sd: *P. palustris*: 11.5 \pm 3.1 cm; *S. laeve*: 0.7 \pm 0.3 cm; *V. americana*: 8.8 \pm 2.3 cm; the reader should note the initial height differences among plant species due to the specificity of each plant functional group; the plant individuals were randomly distributed for the treatments, but we observed a significant initial difference of 0.11 cm between *S. laeve* individuals that received no herbivores (0.70 \pm 0.25 cm) and *S. laeve* individuals that received herbivores (0.59 \pm 0.27 cm) (Supplementary material, Table A1)). Further, *V. americana* individuals growing in mixture not receiving herbivores (9.58 \pm 2.47 cm) were initially 2.58 cm taller by chance than *V. americana* individuals in mixture that received herbivores (7.00 \pm 2.08 cm) (Supplementary material, Table A1). We accounted for these initial height differences in the statistical analyses. In each microcosm, the respective three plants were planted in a triangle of about 5 cm from each other.

Earthworm treatment

Earthworms were ordered at a commercial supplier (easyzoo.de) and handled according to Fründ et al. (2010). We sorted them into juveniles and adults, according to the presence or absence of a clitellum and weighed them (after starving for 24 h at 12°C). The body mass of adults ranged from 2.8 g to 4.6 g (fresh weight of alive earthworms; mean \pm sd: 3.6 \pm 0.5 g), while the weight of juveniles ranged from 1.8 g to 3.5 g (2.6 \pm 0.4 g). Pairs of adults and juveniles were formed to add a similar total body mass of earthworms per microcosm (mean \pm sd: 6.2 \pm 0.12 g). Earthworms were added to half of the microcosms (with earthworm treatment). Four grams of autoclaved (120°C; 90 min) and cut (\sim 1 cm²) litter of poplar species

(*Populus* sp.; C:N= 22.6) sampled in a forest close to Leipzig, was added to each microcosm to feed earthworms and simulate litter conditions in uninvaded Aspen forests.

Herbivore treatment

Herbivores were added in the last week of the experiment and left on the plants for five days in clip cages to prevent them from escaping. Prior to their addition, herbivores were starved for one day and weighed. One clip cage was added to the second (or third if needed) green and healthy leaf per plant, fixing ~50% of it in the cage, and receiving one herbivore each. In total, 141 herbivores were added (three plant individuals were dead at this point in time) to half of the microcosms (with herbivore treatment), and plants without herbivore treatment also received an empty clip cage to control for potential side effects. Clip cages consisted of two foam rings of pipe insulation (inner diameter: ~3 cm, thickness: ~1.5 cm) with mesh of 250 µm closing it on both sides, held together by three hair clips, and fixed by a wire to a wooden stick next to the plant. After herbivore removal, the area consumed by the larvae was visually estimated according to predefined classes (0%, <1%, 1-5%, 6-15%, 16-25%, 26-50%, 51-75%, >76%). The median values of these classes (0, 0.5, 3, 10, 20, 38, 63, 88) were used for further analyses.

Plant trait measurements

Aboveground plant traits were measured for each plant individual. After herbivory rate estimation, ramets were counted, and the height was measured (for *S. laeve* and *P. palustris*, we measured the vegetative height; for *V. americana*, we measured the stretched height due to its growth form). Further analyses were conducted with the height growth values to exclude initial height differences among plant individuals and treatments. To calculate height growth of plants in monoculture, we subtracted the mean initial height per microcosm from the final height of each plant individual. For mixtures, the respective initial height was subtracted from the final height of each individual plant. In microcosms with plant mixtures, inflorescences (only *P. palustris* developed inflorescences) were documented per plant individual, while in

monocultures, they were counted per microcosm, divided by three, and this average value was then used once per (grass) monoculture microcosm for further analysis. Three green and healthy leaves including the petiole (preferably the 3rd, 4th, and 5th youngest) were cut off the plant, weighed, and scanned fresh using a CanoScan LIDE220 Scanner (Canon Inc., Vietnam) at 600 dpi in grayscale. The leaf that was secured in the clip cage (irrespective of herbivore treatment) was previously taken for metabolomic measurements, which are not part of this manuscript. The aboveground biomass was then cut off and stored in paper bags.

To measure belowground plant traits, the soil was removed from the microcosms. Earthworms were collected, starved again, and reweighed after 24 h. Soil samples for pH and water content (each 30 g) analyses were taken and stored at -20°C until further processing. Roots from each plant individual were disentangled and thoroughly washed with water. Debris and black/dead parts of roots were picked out using a tweezer. Roots were sorted into fine roots (<2 mm diameter) and coarse roots (>2 mm diameter). Detached roots were grouped as community roots (including small root pieces from the soil), and the water used for cleaning the roots was sieved (250 µm) to not lose any root material. Further analysis of belowground traits was done on one random individual from plant monocultures and all individuals from plant mixtures. Depending on the mass of the fine roots, either the whole sample or a representative fraction (mean ± sd: 0.6 ± 0.6 g) of it was weighed and scanned using an Epson Perfection 11000XL Scanner (Epson America, Inc., CA, USA) at 600 dpi in grayscale. All samples (aboveground biomass, scanned leaves, and all root samples) were oven-dried at 60°C for 72 h and weighed again to assess their dry mass. Above- and belowground community (microcosm) biomasses were determined by adding the respective dry weights, and community shoot:root ratio was calculated.

Leaf scans were analyzed using the software WinFOLIA (Version: 2014a Pro; Regent Instruments Inc., Canada). Leaf area (cm²) was provided by the software, and we calculated specific leaf area (SLA; leaf area divided by its dry mass (mm² mg⁻¹)), and leaf dry matter content (LDMC; dry mass divided by fresh mass (mg g⁻¹)) using standardized protocols (Pérez-

Harguindeguy et al., 2016). For SLA and LDMC, an average per individual was determined. Root scans were analyzed using the software WinRhizo (Version 2013e Pro, Regent Instruments Inc., Canada), removing objects with an area smaller than 0.001 cm². Larger debris pieces, air bubbles, and scratches were manually excluded. Root average diameter (mm) was provided by the software, and we calculated specific root length (SRL; root length divided by dry mass (m g⁻¹)), root tissue density (RTD; dry mass divided by volume (g cm⁻³)), and root dry matter content (RDMC; dry mass divided by fresh mass (mg g⁻¹)) (Pérez-Harguindeguy et al., 2016; Roumet et al., 2016). For carbon (C) and nitrogen (N) content measurement, leaves of the same plant individual were pooled, and 30 g of soil of each microcosm were analyzed. All samples were dried (60°C for 72 h), ground, and transferred into tin capsules (30 mg, respectively). Analysis was performed using dry combustion with an elemental analyzer (Vario EL cube IR, Elementar Analysensysteme GmbH, Langenselbold, Germany). C and N content were provided as the relative mass proportion of the element (%) per sample mass, and C:N ratio was calculated from these.

Measurements of soil abiotic properties

For the first four weeks of the experiment, one plastic strip with respectively one ion-exchange membrane glued to each side (IEMs, 2.5 cm x 2.5 cm; AMI-7001S and CMI-7000S, Membranes International Inc., Ringwood, NJ, USA) was added to each microcosm at 10 cm depth to assess soil inorganic N availability (Durán et al., 2013). Prior to addition, the membranes were immersed in 5% NaCl solution for 2 h at 37°C to allow for membrane hydration and expansion. After 29 days, anion and cation membrane from IEMs were removed from the soil, air dried, brushed to remove soil particles, and carefully removed from plastic strips. The extraction was performed with 2 M KCL by orbital spinning (1 h at 200 rpm). The extracts were analyzed for ammonium (NH₄⁺), nitrate (NO₃⁻) by the indophenol blue method (Sims et al., 1995) and phosphate (PO₄³⁻) by the molybdenum blue method (Allen et al., 1986), with a microplate reader (Durán et al., 2008). One sample was excluded from statistical analysis, as we only found the plastic strip at the end of the experiment and not after 29 days

(Grass-Control-2). For pH measurements, 10 g of air-dried soil was solved in 25 ml of 0.01 M CaCl_2 solution, shaken, and left for 1 h. Measurements were conducted with a pH-meter (Orion Star A211, Thermo scientific, MA) according to the manual. To determine soil water content, fresh soil was weighed, dried in a drying oven (75°C for at least 24 h), and reweighed.

Statistical analyses

Statistical analyses and figures were conducted and created, respectively, using the R software version 4.1.2 (R Core Team, 2021). Each trait was analyzed using a linear model, followed by a Type II ANOVA from the package 'car' (Fox & Weisberg, 2019) after removing missing data (NAs). Plant species identity, plant diversity level, earthworm treatment, and herbivore treatment were analyzed as factors in a four-way interaction, respectively. Assumptions of the models were visually checked using the R packages 'performance' (Lüdecke et al., 2021a) and 'see' (Lüdecke et al., 2021b). When needed, variables were log transformed, such as aboveground biomass, leaf area, LDMC, SLA, SRL, RTD, nitrogen content of leaves, soil water content, herbivory rate, PO_4^{3-} , and NH_4^+ of soil. To analyze height growth and aboveground biomass, the averaged initial height was added to the model as a covariate, but not in interaction with the other factors. For count (ramets) and proportion (survival data) data, generalized linear models (Poisson and binomial, respectively) were performed using the package 'stats' (R Core Team, 2021). As only *P. palustris* developed inflorescences, species identity was not considered for the analysis. When analyzing herbivore survival/rate or earthworm survival the respective treatments (*i.e.* herbivore and/or earthworm presence) were excluded as an explanatory variable. For community traits and soil abiotic measurements the species column included the necessary information (*S. laeve*, *P. palustris*, *V. americana*, or mixture) and the plant diversity level was therefore excluded as an explanatory variable. Post-hoc tests of the significant interactions were conducted using the function 'emmeans' from the correspondent R package (Lenth, 2021) with Holm correction. Compact letter displays were created using the R package 'multcomp' (Hothorn et al., 2008). Data were plotted per trait using 'ggplot2' (Wickham, 2016) and the letters correspond to the

significant interaction with the highest number of factors. For calculating the change of traits in percentage the estimated marginal means from the post-hoc tests were used. All results are in SI and the following abbreviations are used for the statistical effects in the results: earthworm treatment= EW; herbivore treatment= Hrb; plant species identity= Spc; Plant diversity level= Clt.

Results

Plant community productivity responses

Only five out of the 288 plant individuals died during the experiment (1.7%), four of which were *V. americana* individuals growing in mixture and one *S. laeve* from a monoculture (Supplementary material, Table A2). In the presence of earthworms, *P. palustris* and mixed communities produced significantly more aboveground biomass than when earthworms were absent (*P. palustris*: +26%, mixed community: +23%; EW:Spc interaction effect: $p=0.0006$; Figure 1; Supplementary material, Table A3). Root community biomass and community shoot:root ratio were not significantly affected by earthworm presence (Supplementary material, Table A3, Figure A1 and A2).

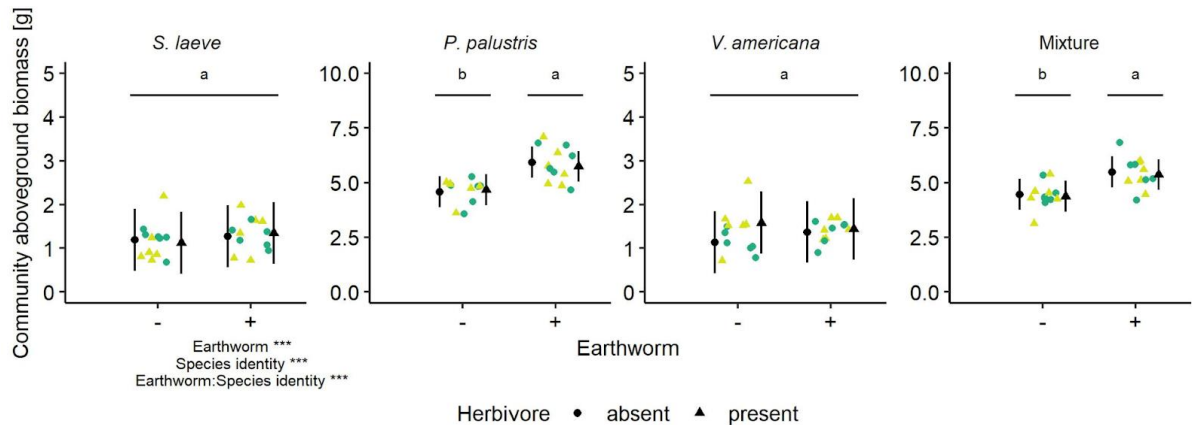


Figure 1: Community aboveground biomass (per microcosm) (estimated marginal mean \pm CI_{95%}) of different plant communities (monocultures of *Symphyotrichum laeve*, *Poa palustris*, and *Vicia americana*, or mixture of all three species) with (+) and without (-) earthworm as well as herbivore treatment (presence (yellow triangles) vs. absent (green circles)). A linear model was applied to analyze these data after excluding NA's. The letters were derived from a post-hoc test (pairwise comparison of estimated marginal means using the "holm" correction) investigating

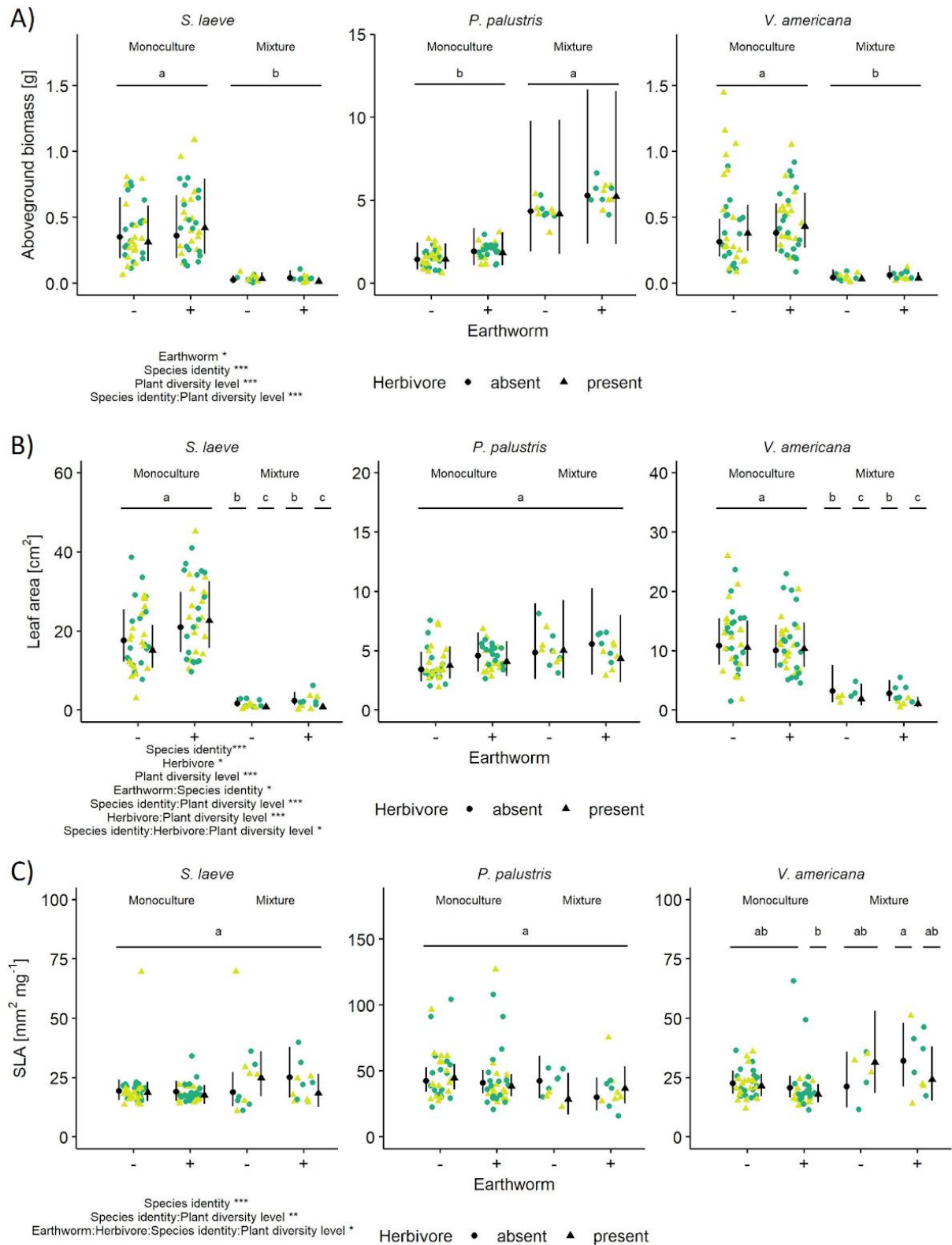
the effect of earthworm treatment for each plant community. Different letters represent a significant difference (p-value < 0.05) and can be interpreted only within each community. In total, 96 observations were analyzed (each community type: 24; earthworm and herbivore absence/presence: 48 each). Significant factors are displayed in the bottom left corner (**** 0.001; *** 0.01; ** 0.05).

Plant functional trait responses

All studied plant functional traits differed significantly among plant species. Eight out of eleven plant functional traits were significantly affected by earthworm presence; either by a general effect across all treatments or the effect differed significantly among plant species, with plant diversity level, and/or herbivore presence.

Aboveground trait responses

Among the aboveground traits measured, earthworm treatment overall affected aboveground biomass, leaf area, specific leaf area, and leaf dry matter content, but not the height growth (Supplementary material, Table A4). Earthworm presence increased aboveground biomass across all plant species (+15%), irrespective of other treatments (EW effect: p=0.017). Overall, *V. americana* and *P. palustris* individuals had the largest height difference between initial and final height (*V. americana*: 22.10 ± 11.39 cm, *P. palustris*: 16.55 ± 5.60 cm), while *S. laeve* individuals grew the least (3.80 ± 2.73 cm; Supplementary material, Figure A3). Earthworm and herbivore treatments did not significantly affect the height growth of any plant species. *Symphyotrichum laeve* and *P. palustris* individuals had a higher leaf area when earthworms were present (*S. laeve*: +22%, *P. palustris*: +9%), while it was the opposite for *V. americana* individuals (-18%; EW:Spc interaction effect: p=0.043). Despite leaf area changes, the SLA of *S. laeve* and *P. palustris* were not significantly affected by the experimental treatments. However, in the presence of earthworms, the SLA of *V. americana* was higher in the absence of herbivores in mixture in comparison to the treatment with herbivores in monoculture (-44%), while the other treatment combinations had a similar SLA (EW:Hrb:Clt:Spc interaction effect: p=0.013; Figure 2; Supplementary material, Table A4). The LDMC was generally higher (+9%) when earthworms were present (EW effect: p=0.006; Supplementary material, Figure A4).



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Figure 2: Aboveground biomass (A), leaf area (B), and specific leaf area (C) (estimated marginal mean \pm CI_{95%}) of the three plant species studied (*Symphyotrichum laeve*, *Poa palustris*, and *Vicia americana*) growing in different plant diversity levels(monoculture vs. mixture) with (+) and without (-) earthworm as well as herbivore treatment (presence (yellow triangles) vs. absent (green circles)). A linear model with a log transformation was applied to

analyze these data after excluding NA's. Data were back-transformed before plotting. The letters were all derived from post-hoc tests (pairwise comparison of estimated marginal means using the "holm" correction). Different letters represent a significant difference (p -value < 0.05) and can be interpreted only within each species. Significant factors are displayed in the bottom left corners (**** 0.001; *** 0.01; ** 0.05). A) Post hoc tests investigated the effect of plant diversity level for each species. In total, 286 observations were analyzed (*S. laeve*: 95; *V. americana*: 95; *P. palustris*: 96; monoculture: 215; mixture: 71; earthworm and herbivore absence/presence: each 143). B) Post hoc tests investigated the interaction between herbivore treatment and plant diversity level for each species. In total, 277 observations were analyzed (*S. laeve*: 93; *P. palustris*: 96; *V. americana*: 88; monoculture: 214; mixture: 63; earthworm absence: 137; earthworm presence: 140, herbivore absence: 139; herbivore presence: 138). C) Post hoc tests investigated the interaction of plant diversity level, earthworm, and herbivore treatment for each species. In total, 272 observations were analyzed (*S. laeve*: 93; *V. americana*: 87; *P. palustris*: 92; monoculture: 214; mixture: 58; earthworm absence: 138, earthworm presence: 134, herbivore absence: 137; herbivore presence: 135).

Earthworms affected the C, N, and C:N ratio of the leaves (Supplementary material, Table A4). The C content of *S. laeve* leaves growing in mixture without earthworms was higher compared to individuals growing in monoculture irrespective of earthworm presence (+6%; EW:Clt:Spc interaction effect: $p=0.006$). The leaf N content of *P. palustris* was increased in the presence of earthworms (+27%), while no significant difference was found for the other two species (EW:Spc interaction effect: $p=0.0004$). *Symphyotrichum laeve* and *P. palustris* both had a lower C:N ratio when earthworms were present (*S. laeve*: -9%; *P. palustris*: -19%; EW:Spc interaction effect: $p<0.0001$; Figure 3). Across all species, plant individuals growing in mixture without earthworms had the highest C:N ratio, and plant individuals growing in monoculture with earthworms present the lowest (EW:Clt interaction effect: $p=0.040$). The C:N ratio of plant individuals growing in mixture with earthworms did not differ significantly from plant individuals growing in monoculture without earthworms, and their C:N ratios were between the other two plant diversity level/ earthworm treatment combinations (Figure 3). Overall, plant individuals growing in monocultures with earthworms had a lower C:N ratio (-7%) than the ones without earthworms. Plant individuals growing without earthworms had a higher C:N ratio in mixtures (+18%) than in monocultures.

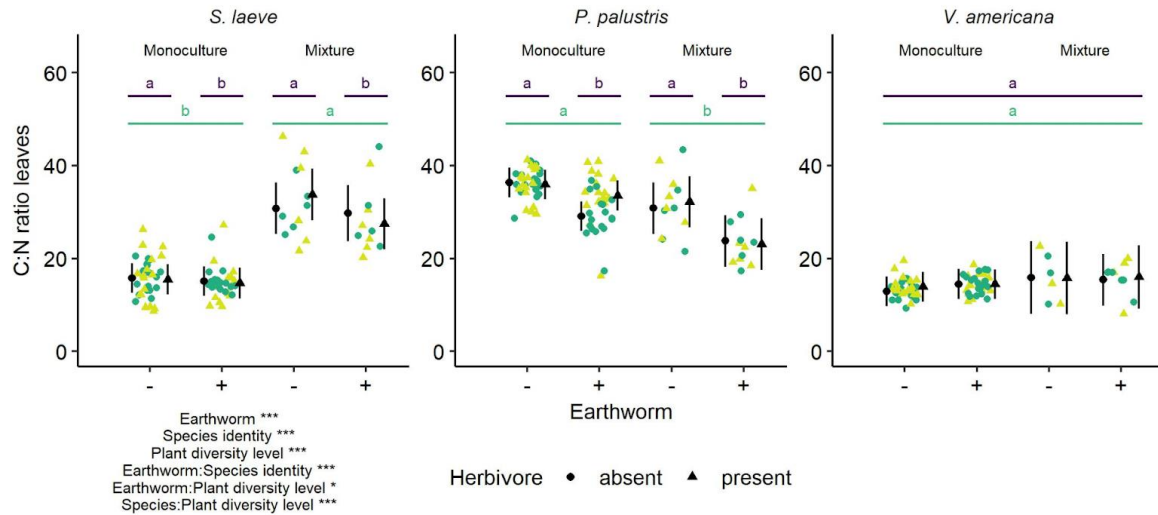


Figure 3: C:N ratio of leaves (estimated marginal mean \pm CI_{95%}) of the three plant species studied (*Symphytotrichum laeve*, *Poa palustris*, and *Vicia americana*) growing in different plant diversity levels (monoculture vs. mixture) with (+) and without (-) earthworm as well as herbivore treatment (presence (yellow triangles) vs. absent (green circles)). A linear model was applied to analyze these data after excluding NA's. The letters were derived from post-hoc tests (pairwise comparison of estimated marginal means using the "holm" correction). The purple letters (upper) come from a post-hoc investigating the effect of earthworm treatment for each species. The green letters (lower) were derived from a post-hoc test investigating the effect of plant diversity level for each species. Different letters represent a significant difference (p-value < 0.05) and can be interpreted only within each species. In total, 278 observations were analyzed (*S. laeve*: 94; *V. americana*: 88; *P. palustris*: 96; monoculture: 215; mixture: 63; earthworm absence: 138; earthworm presence: 140; herbivore absence: 140; herbivore presence: 138). Significant factors are displayed in the bottom left corner (**** 0.001; *** 0.01; ** 0.05).

Reproductive trait responses

While only *P. palustris* individuals developed inflorescences that were produced in higher number in mixtures (+172%; Clt effect: $p < 0.0001$) and when earthworms were present (+17%; EW effect: $p = 0.011$; Supplementary material, Figure A7, Table A5), vegetative reproductive abilities (*i.e.* number of ramets) were overall increased for all species in the presence of earthworms (+12%; EW effect: $p < 0.0001$; Supplementary material, Figure A8, Table A5). Moreover, *Poa palustris* produced more ramets in plant mixtures (+124%), while *V. americana* had a lower number of ramets in mixtures (-36%; Spc:Clt interaction effect: $p < 0.0001$).

Belowground trait responses

We found coarse roots for *V. americana* (46 samples) and *S. laeve* (four samples), while *P. palustris* only had fine roots. Only root average diameter and root tissue density measured on the fine roots samples varied in response to the earthworm treatment, the herbivore treatment, and the plant species (Figure 4; Supplementary material, Table A6). Root dry matter content (RDMC) was higher in mixture compared to monoculture (+54%; Clt effect: $p=0.018$), but was not affected by earthworm presence nor another treatment (Table 3; Supplementary material, Figure A9). In the same way, plant diversity level, earthworm presence, and herbivore presence did not significantly affect specific root length (Supplementary material, Table A6). However, root average diameter of *S. laeve* individuals growing in mixture with either earthworms present and herbivore absent, or earthworms absent and herbivore present was lower (-30%) than that of the same species in monoculture with the same treatment combinations (EW:Hrb:Clc:Spc interaction effect: $p=0.033$; Figure 4), while the other plant species were not significantly affected by this interaction of the experimental treatments. The roots of *V. americana* individuals had a lower root tissue density (RTD) when earthworms were present compared to the treatment without earthworms (-21%; Spc:EW: $p=0.015$; purple letters in Figure 4). In the absence of earthworms, all three studied plant species had higher RTD when herbivores were present (+23%) compared to the treatment without herbivores (EW:Hrb interaction effect: $p=0.009$).

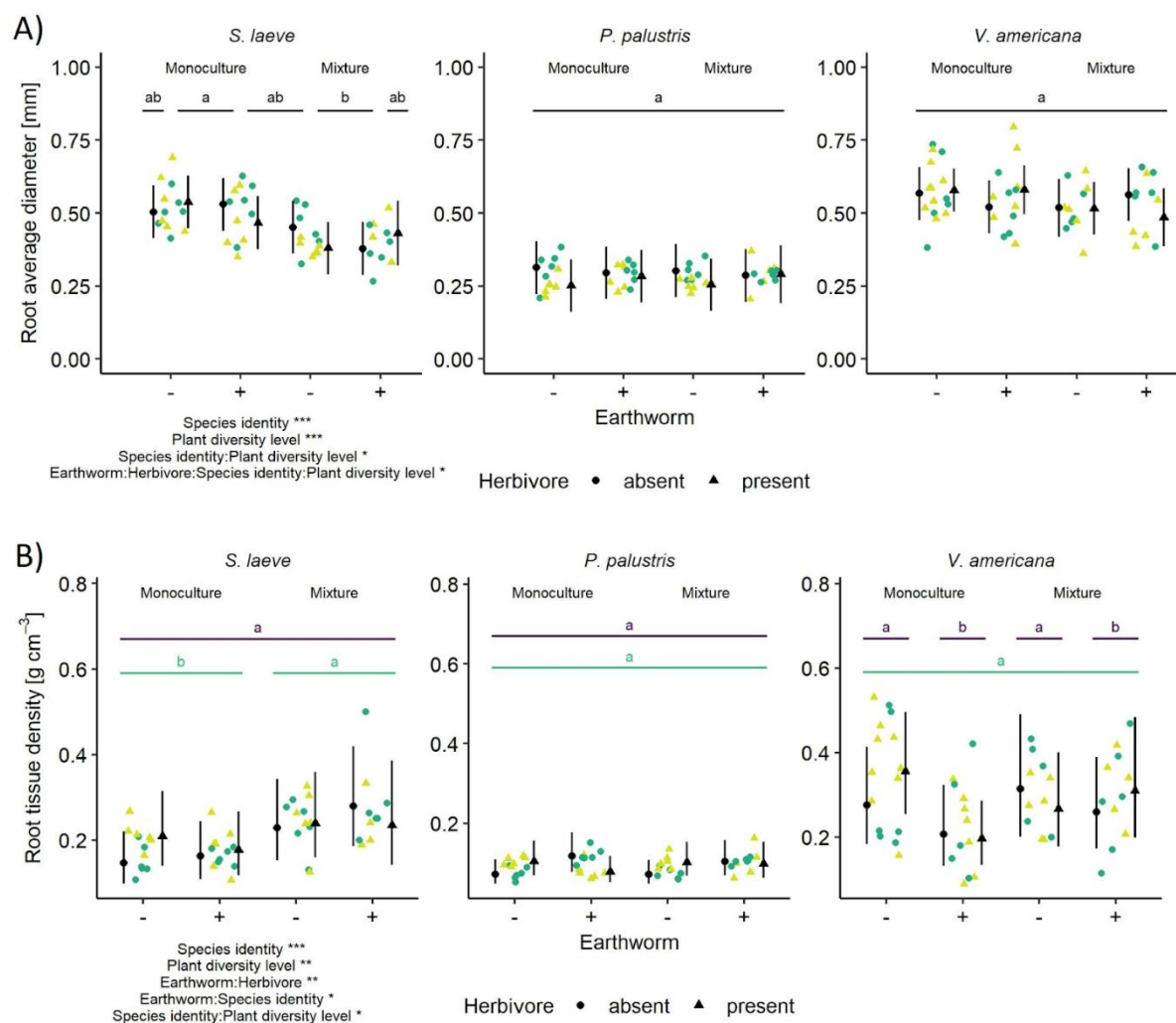


Figure 4: Root average diameter (A) and root tissue density (B) (estimated marginal mean \pm CI_{95%}) of the three plant species studied (*Symphyotrichum laeve*, *Poa palustris*, and *Vicia americana*) growing in different plant diversity levels (monoculture vs. mixture) with (+) and without (-) earthworm as well as herbivore treatment (presence (yellow triangles) vs. absent (green circles)). After excluding NA's, a linear model was applied to analyze data. The letters were all derived from post-hoc tests (pairwise comparison of estimated marginal means using the "holm" correction). Different letters represent a significant difference (p-value < 0.05) and can be interpreted only within each species. Significant factors are displayed in the bottom left corner (**** 0.001; *** 0.01; * 0.05), respectively. A) Post hoc tests investigated the interaction of plant diversity level and earthworm and herbivore treatment for each species. In total, 143 observations were analyzed (*S. laeve*: 46; *V. americana*: 47; *P. palustris*: 50; monoculture: 76; mixture: 67; earthworm absence: 69; earthworm presence: 67; herbivore absence: 71; herbivore presence: 72). B) Data was log transformed before analysis and back-transformed before plotting. The purple letters (upper) come from a post-hoc investigating the effect of earthworm treatment for each species. The green letters (lower) were derived from a post-hoc test investigating the effect of plant diversity level for each species. In total, 142 observations were

analyzed (*S. laeve*: 46; *V. americana*: 49; *P. palustris*: 47; monoculture: 75; mixture: 67; earthworm absence: 74; earthworm presence: 68; herbivore absence: 70; herbivore presence: 72).

Earthworm / herbivore survival and herbivory rate

Out of the 96 earthworms added to experimental microcosms, 68 were found alive at the end of the experiment (70%), and no experimental treatment influenced their survival significantly (Supplementary material, Table A2). We also found that 118 herbivores died or escaped (84%) after five days, while 23 were still alive and present (16%), and 97 out of 141 leaves (64%) had marks of herbivory on them. Most herbivores survived on *S. laeve* growing in monoculture (44%) followed by *V. americana* monoculture (17%), while no herbivores survived on these plant species growing in mixture. One herbivore survived on *P. palustris* growing in mixture, none when growing in monoculture (Supplementary material, Table A2). Overall, the *S. exigua* larvae fed most on *S. laeve*, secondly on *V. americana*, and the least on *P. palustris* (Spc effect: $p=0.017$; Supplementary material, Table A7, Figure A11). Earthworm presence had no significant effect on the herbivory rate.

Soil abiotic properties

Soil pH (7.41 ± 0.05) and soil C:N ratio (17.65 ± 3.67) were not significantly affected by the experimental treatments (Supplementary material, Table A8, Figure A12 and A13). Soil water content was significantly lower (-9%) in the presence of earthworms (EW effect: $p=0.027$; Supplementary material, Table A8, Figure A14). Soil C content did not differ among plant species, but soil of *P. palustris* communities contained less C when earthworms were present (-19%; EW:Spc interaction effect: $p=0.013$; Supplementary material, Table A8, Figure A15), and soil of *Vicia americana* communities had an increased soil C content in the presence of herbivores (+17%; Hrb:Spc interaction effect: $p=0.012$). Soil of *P. palustris* communities contained significantly more N without earthworms and herbivores present (+48%) compared to other treatment combinations (EW:Spc:Hrb interaction effect: $p=0.041$). Within the first four weeks of the experiment, no other factor apart from plant species identity had a significant

effect on PO_4^{3-} (Spc effect: $p=0.0003$; Supplementary material, Table A8, Figure A17). In presence of earthworms, soil NO_3^- increased significantly (+54%; EW effect: $p=0.0003$; Supplementary material, Table A8, Figure A18). NH_4^+ did not change significantly in response to any treatment, including plant species identity and earthworm treatments (Supplementary material, Table A8, Figure A19).

Discussion

Earthworms change plant trait expression

Given that earthworm invasion is changing plant communities in North American forests towards lower diversity and grass dominance (Craven et al., 2017), we investigated the underlying mechanisms by examining how earthworms affect the functional traits of plant species belonging to three different plant functional groups (*i.e.* grasses, herbs, and legumes) in different plant communities (*i.e.* different levels of competition) and in the presence and absence of aboveground herbivores. Our study confirms that invasive earthworms can affect plant functional traits of native plants in a significant way, which is in line with the recent findings of Thouvenot et al. (2021) and Blume-Werry et al. (2020). We observed that eight out of eleven measured plant functional traits were affected either by the main effect of earthworms, or earthworm effects in combination with those of plant species identity, plant diversity level, and/or herbivore presence. Only height growth, root dry matter content, and specific root length did not respond significantly to earthworm presence. Across all plant species, earthworm treatment (alone) increased the number of ramets, the LDMC, and induced a modification in the leaf area. For example, we found that *V. americana* developed a smaller leaf area, while *S. leave* and *P. palustris* developed a larger leaf area when growing in earthworm presence. These changes might be based on higher nutrient availability due to the activities of earthworms (van Groenigen et al., 2014). Indeed, it was shown that LDMC is a predictor for soil fertility (Hodgson et al., 2011), and Zaller & Arnone (1999) explained increased ramet production close to earthworm casts mainly by enhanced nutrient availability. This is in line

with increased concentrations of nitrate in soil and higher leaf nutritional value in *S. laeve* (lower C:N) and *P. palustris* (lower C:N; higher N) in the presence of earthworms. The observed increase of soil nitrate in the presence of earthworms supports the assumption that earthworms enhance soil nitrogen availability and uptake by plants (van Groenigen et al., 2014).

Grass aboveground productivity and reproduction benefits from interspecific competition and earthworms

Aboveground productivity of *P. palustris* monocultures and the mixed community were significantly increased in the presence of earthworms, while productivity of *S. laeve* and *V. americana* communities did not show any general short-term responses to the earthworm treatment. These results support the findings that slow-growing species cannot exploit additional nutrients as rapidly as fast-growing species (Reich, 2014), and provide only partial support for our hypothesis (1), expecting that plant productivity is generally increased by earthworm presence. Grass community productivity may be more responsive to earthworm activity due to their high resource competitiveness and rapid growth (Eisenhauer & Scheu, 2008a; Linder et al., 2018; Thouvenot et al., 2021).

When growing in interspecific competition, several traits of *P. palustris* (lower C:N, more aboveground biomass, ramets, and inflorescences) indicate an elevated nutrient uptake, growth, and reproduction. Reduced SLA points to decreased photosynthetic rate (Violle et al., 2007), probably due to less competition for light. Moreover, *P. palustris* developed more inflorescences when growing in earthworm presence. Inflorescences are an indicator for individual plant performance (Roscher et al., 2011), and an increased sexual reproduction may represent a competitive advantage (E-Vojtkó et al., 2020) rarely reported in previous studies on the consequences of earthworm invasion (Blume-Werry et al., 2020). As earthworms can change nutrient and water availability (Ferlian et al., 2020), they might act as a filter on plant reproduction (Eisenhauer et al., 2012; E-Vojtkó et al., 2020). Even though we found that *P. palustris* individuals benefit from earthworm presence and from interspecific competition (or

missing intraspecific competition), we did not find an interaction effect of these two factors. Besides several functional traits of the legume species *V. americana* and the herb species *S. laeve* changing in interspecific competition (e.g. lower aboveground biomass, lower height growth, less ramets), four out of 24 *V. americana* individuals died that grew in mixed communities. The legume individuals developed a higher SLA in interspecific competition, which is a strategy to cope with lower light availability by raising their photosynthetic rate (Roscher et al., 2011; Violle et al., 2007). Nevertheless, we did not find an interaction effect of plant diversity level and earthworm presence for any of the studied traits for these two species, providing little support for our hypothesis (2). The finding that neither earthworm presence, nor a change of plant diversity level had a significant effect on the C:N ratio of *V. americana* leaves, supports results of former studies that state that legumes are rather independent of soil nutrient changes, e.g. caused by plant competition or the presence of earthworms (van Groenigen et al., 2014; Wurst et al., 2003). Since *S. laeve* and *V. americana* did not develop inflorescences in any of the treatments, it seems that the experiment was too short for this to happen. As seed production is often referred to as “the fitness currency” of plants (Schwachtje et al., 2006) and flowering is a critical prerequisite, the effects of earthworms on flowering and seed production deserve more attention, and experiments carried out over a longer period of time are needed. Moreover, such studies would help finding out whether the grasses only developed inflorescences earlier or more overall when earthworms were present.

Earthworm presence does not affect herbivory rate or herbivore survival

Neither herbivory rate, nor herbivore survival, of any of the plant species was affected by earthworm presence (Hypothesis (4)). As the initial C:N ratio of the soil used (20.98) was higher and the N% (0.06%) lower than in comparable studies (Eisenhauer et al., 2007; Thouvenot et al., 2021; Wurst et al., 2005), we do not expect the lack of earthworm effects on herbivory and herbivore survival to be due to a too high initial soil nutrient status (van Groenigen et al., 2014). Contrary to the expectations that slow-growing species have a better defense than fast-

growing species (Reich, 2014), the grass species studied was not only the least palatable, but also the least suitable for the survival of herbivores. A possible explanation is that grass leaves contain silica, which enhances the tissues abrasiveness and reduces its digestibility (Luyckx et al., 2017; Vicari & Bazely, 1993) by changing leaf surface morphology (Hall et al., 2020). The increase of LDMC (higher defense; all plant species) and the decrease of C:N ratio (higher nutritious value; *P. palustris* and *S. laeve*) caused by earthworms could also have balanced each other out and, thus, prevented a change of herbivory rate. Furthermore, multiple aspects that have an impact on the outcome of earthworm effects on herbivory have been identified, which may also differ with the environmental context. For example, it was shown that the density and diversity (species and ecological groups) of earthworms, the herbivore feeding guilds (chewing, phloem-feeding, cell-feeding), and plant functional group identity may play a role (Xiao et al., 2018).

Earthworm presence and competitive environment modulate herbivore effects on specific traits

We found effects of short-term aboveground herbivory on plant traits (*i.e.* LDMC, leaf area, RTD; hypothesis (3)). However, the effects were modulated by interspecific competition and earthworm presence, supporting our hypothesis (5) (*i.e.* effects of invasive earthworms and aboveground herbivory depend on plant community composition). Leaf traits (LDMC and leaf area) of plants growing in interspecific competition changed in the presence of herbivores, which indicates that interspecific competition led to the leaf development being more sensitive to herbivory, and plants invested more into mechanical defense (tougher leaves) (Loranger et al., 2012).

In the absence of earthworms, herbivore presence led to a higher RTD across plant species, which is associated with higher resistance to pathogens and drought (Pérez-Harguindeguy et al., 2016). This might offer an advantage in tolerating herbivore attack by reducing the risk of other influences that may harm the herbivory-weakened plant (e.g. drought and pathogens). Further, a higher RTD might indicate translocation of resources after herbivore attack (Babst

et al., 2008; Gómez et al., 2010; Schwachtje et al., 2006) or a more conservative use of, for example, carbon through lower root exudation (Wen et al., 2021). Vice versa, our data shows that the RTD of plants that grew with earthworms did not change in response to herbivory, indicating that earthworms suppressed an herbivory-induced increase of RTD. Herbivory rate was not lower when earthworms were present. Possibly, firmer leaf tissue (measured as higher LDMC) might have reduced the effect of herbivory on the plants' condition and thus did not cause any significant change of RTD.

The effects of earthworm presence on the root average diameter of *S. laeve* and to a lesser extent on the SLA of *V. americana* were also dependent on the biotic context. The similar effect of earthworms and herbivores, respectively, in the absence of the other leading to the highest and lowest values of root average diameter within the plant diversity level is surprising, since they have fundamentally different effects on plants. Presumably, herbivory led to a redistribution of resources towards roots (e.g. Schwachtje et al., 2006), while earthworms are known to improve nutrient availability for plants (Scheu, 2003; van Groenigen et al., 2014). Changed resource availability due to competition (Aschehoug et al., 2016) modulated these effects, but when earthworms and herbivores were both present, root average diameter changes were not observed, regardless of plant diversity levels. These complex interactions for the first time indicate that earthworms interact with interspecific competition and herbivory. We recommend for future studies to investigate the mechanisms behind these interactions on belowground traits for different plant species to be able to predict the consequences for plant community structure. Further, we suggest studying additional root resource-acquisition strategies, such as mycorrhizal symbiosis and root exudation, since these may also play critical roles in plant resource use and competition (Wen et al., 2021). To relate all our results more broadly to the functional groups of plants, we recommend testing more and different plant species per plant functional group in future studies.

Conclusions

Our study provides experimental evidence that invasive earthworms affect plant resource acquisition and competitive abilities by inducing changes in above- and belowground plant functional traits. These effects were partly modulated by competition and herbivory, and may reveal some of the mechanisms behind plant community changes in northern North American forests after earthworm invasion. Earthworms did not only enhance the aboveground biomass of the grass *P. palustris* but also improved its competitive advantage, such as reflected by a higher number of inflorescences, enhanced leaf area, and elevated tissue N content. Our findings thus confirm that the grass species *P. palustris* is a strong competitor and benefits from earthworm presence at least in the short term. The herb and legume species did not benefit to a similar extent by earthworm presence, which is why we assume that earthworms increased the competitive strength of the grass species at the expense of the other plant functional groups. Earthworms did not affect herbivory rate and herbivore survival, but suppressed an effect of herbivores on the root tissue density. Our study shows that changed plant trait expression by earthworms is partly depending on the biotic context of the plants; *i.e.* competition and herbivory. Taken together, the results of the present study thus shed light on trait-based mechanisms potentially underlying observed shifts in plant community composition in northern North American forests to the benefit of grasses and the expense of legumes.

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