Interactive effects of global warming and 'global worming' on the initial establishment of native and exotic herbaceous plant species

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The spread of exotic earthworms ('worming') and rising temperatures are expected to alter the biological, chemical and physical properties of many ecosystems, yet little is known about their potential interactive effects. We performed a laboratory microcosm experiment to investigate the effects of earthworms (anecic, endogeic, epigeic, or all three together) and 4°C warming on soil water content, litter turnover and seedling establishment of four native and four exotic herbaceous plant species.

Warming and worming exerted independent as well as interactive effects on soil processes and plant dynamics. Warming reduced the water content of the upper soil layer, but only in the presence of earthworms. Litter removal increased in the presence of earthworms, the effect being most pronounced in the presence of anecic earthworms at ambient temperature. Exotic plant species were most influenced by earthworms (lower seedling number but higher biomass), whereas natives were most sensitive to warming (higher seedling number). This differential response resulted in significant interaction effects of earthworms and warming on abundance and richness of native relative to exotic plants as well as related shifts in plant species composition. Structural equation modeling allowed us to address possible mechanisms: direct effects of earthworms primarily affected exotic plants, whereas earthworms and warming indirectly and differentially affected native and exotic plants through changes in soil water content and surface litter.

Invasive earthworms and warming are likely to interactively impact abiotic and biotic ecosystem properties. The invasion of epigeic and anecic species could select for plant species able to germinate on bare soil and tolerate drought, with the latter becoming more important in a warmer world. Thus earthworm invasion may result in simplified plant communities of increased susceptibility to the invasion of exotic plants.

Humankind is changing ecosystems worldwide by biotic exchange and alterations in climatic conditions (Vitousek et al. 1996, Millenium Ecosystem Assessment 2005). One target of such change is the herbaceous layer, which is crucial for the functioning of forest ecosystems (Gilliam 2007). The spread of exotic ecosystem engineers has profound impacts on the abiotic and biotic properties of native ecosystems (Bohlen et al. 2004a, Hendrix et al. 2008). Although belowground invasions often take place unperceived, the invasion of European earthworms into North American ecosystems has received increasing attention (Bohlen et al. 2004a, Frelich et al. 2006, Hendrix et al. 2008). Exotic earthworms have been shown to change soil structure (e.g. thickness of organic soil layers; Hale et al. 2005a, 2008, Eisenhauer et al. 2007), soil chemical properties and nutrient cycling (e.g. pH, carbon, nitrogen and phosphorous content; Bohlen et al. 2004b, Hendrix et al. 2008), soil microbial communities and functions (McLean et al. 2006, Szlavecz et al. 2011), and plant community composition (Hale et al. 2006, Holdsworth et al. 2007) of native habitats.

Exotic earthworms can directly or indirectly affect critical life stages of herbaceous plant species via 1) reduced reproduction and survival due to seed and seedling consumption as well as seed burial, 2) alterations in seed bank composition and germination microclimate, and 3) elevated susceptibility to vertebrate herbivory (Bohlen et al. 2004a, Eisenhauer et al. 2009a). Moreover, there is increasing awareness that invasion of exotic earthworms and plants may be linked (Corio et al. 2009, Madritch and Lindroth 2009, Nuzzo et al. 2009). The degree of invasion of exotic plant species has been suggested to be a function of the degree of anthropogenic disturbance, with undisturbed forests being more resistant to invasion (reviewed by Gilliam 2007). Habitat modifications induced by exotic earthworms apparently represent major disturbances for many ecosystems, hence probably facilitating subsequent plant invasions.

The impact of earthworm invasion on native ecosystems depends on the earthworm ecological group (Bohlen et al. 2004a, Hendrix et al. 2008). Epigeic earthworms reside mainly in the upper organic soil layers and cause limited mixing of mineral and organic layers by feeding on litter materials. They have little to moderate effects on litter layer thickness, nutrient cycling and microbial functions (Scheu and Parkinson 1994, Hale et al. 2005b). Endogeic earthworms

live in the upper mineral soil, primarily consume mineral soil materials and form horizontal, non-permanent burrows. They can have strong effects on soil abiotic and biotic properties by compacting the soil and changing soil nutrient status (Eisenhauer et al. 2007, Straube et al. 2009). Anecic earthworms are intermediate between litter-dwelling epigeic and soil-dwelling endogeic species in that they feed on litter but live in burrows in soil. They form vertical permanent burrows up to 2 m deep and incorporate litter from the soil surface into deeper soil layers. Anecic earthworms are well-known to remove the litter layer when they invade forest ecosystems (Hale et al. 2005a). In addition, they can act as seed and seedling predators with major effects on plant community composition (Eisenhauer et al. 2010).

Increasing global temperatures will change the composition and functioning of ecosystems (Harte and Shaw 1995, Melillo et al. 2002, Lambrecht et al. 2007) and probably the outcome of competition between native and exotic plant species, by benefiting species adapted to higher temperatures and drought. Seed germination and seedling establishment are bottleneck stages in the life cycle of plants (Körner 2003), and are sensitive to environmental changes like warming (Lloret et al. 2009, Milbau et al. 2009, Shevtsova et al. 2009, Walck et al. 2011) and earthworm invasion (Eisenhauer et al. 2009a). The underlying mechanisms are multifaceted. Plant germination may respond in a species- and context-dependent way to rising temperatures, and accompanying changes in other microclimatic conditions, such as drought, reduced severity of frost and extension of the growing season (Milbau et al. 2009, Shevtsova et al. 2009). Earthworms are known to bury, ingest, egest and digest seeds, as well as change seedbed conditions (McRill and Sagar 1973, Eisenhauer et al. 2009a, b). As a result, earthworms tend to decrease the number of seedlings, while increasing individual seedling biomass (Milcu et al. 2006, Eisenhauer and Scheu 2008), which likely results in higher survival rates (Eisenhauer et al. 2008a). Moreover, earthworms may decrease soil water content as their burrows represent preferential flow pathways (Shipitalo et al. 2004). Despite the pivotal consequences of warming and earthworm invasion for the germination and establishment of seeds and seedlings, interactive effects of these anthropogenic change agents have, to our knowledge, not been investigated before. Indeed, there is evidence that warming and soil disturbances similar to those induced by exotic earthworms interactively affect seedling establishment in black spruce (Munier et al. 2010). For instance, potential detrimental effects of warming on seedling establishment through water stress could be exacerbated by anecic earthworms removing the litter layer and creating preferential flow pathways. Thus, interactions between warming and invasive earthworm may potentially affect competition between native and exotic plants species.

Here, we studied the effects of exotic earthworms belonging to three ecological groups (anecic, endogeic and epigeic; Bouché 1977) and environmental warming on seedling establishment of four native and four exotic plant species in a microcosm experiment. We used representative plant and earthworm species naturally co-occurring in a global change experiment in northern Minnesota, USA (B4WarmED experiment). We hypothesized that 1) anecic and epigeic earthworms decrease seedling number and species richness due to seed burial and ingestion as well as a decrease in soil water content due to preferential water flow and reduced litter coverage; 2) warming decreases the number and biomass of seedlings due to reduced soil water content; 3) warming and earthworms interactively affect seedling establishment due to distinct non-additive mechanisms. For instance, warming could decrease the activity and thus the effect of earthworms, whereas earthworms may intensify the soil moisture-related effect of warming by removing soil surface litter and building preferential flow pathways for surface water. 4) Warming and earthworms shift the plant community towards the exotic species because these may be better pre-adapted than the natives to the impact of earthworm activities and increased temperatures. This hypothesis stems from the observation that the exotic plant species used in the experiment are common in temperate European grasslands that likely experience higher temperatures and have minimal litter depth, compared to the temperate forests they have colonized in North America.

Material and methods

Experimental setup

We set up microcosms consisting of PVC tubes (inner diameter 10 cm, height 25 cm) covered by a 5 mm mesh at the bottom to allow drainage of water. A plastic barrier (10 cm height) at the top of the microcosm reduced the escape of earthworms from experiment containers. The soil (pH 5.76, carbon concentration 1.57%, nitrogen concentration 0.091%, C-to-N ratio 17.2; water content 15%) was taken from the field site of the B4WarmED experiment at the Cloquet Forestry Center, (Cloquet, MN, USA). The B4WarmED experiment is a field study established in 2008 investigating the effects of warming on tree competition and ecosystem functioning in open and closed canopy habitats. Numerous exotic Eurasian herbaceous plant species are established in the open habitats. Both habitats have been invaded by exotic earthworms; the most abundant species are *Lumbricus rubellus* (epi-endogeic) and *Aporrectodea caliginosa* (endogeic), while *Lumbricus terrestris* (anecic) has also invaded some adjacent forest stands.

A total of 60 microcosms were filled with 5 cm of perlite at the bottom to allow drainage of water and prevent the escape of earthworms and then with 2 kg (fresh weight; total height of the soil column 20 cm) of sieved (1 cm) and homogenized soil. The microcosms were placed in six temperature controlled growth chambers at a day/night regime of 16/8 h. Three growth chambers with 10 pots each were kept at 16/12 ± 1°C (ambient; mean temperature 14.67°C) and the other three chambers with 10 pots each were kept at 20/16 ± 1°C (+ 4°C; mean temperature 18.67°C). The ambient treatment corresponded to the mean temperature of the growing season (~April to October) at the Cloquet experimental field site from 2008 to 2010, whereas the + 4°C treatment simulated warming in the range expected by the end of this century (IPCC 2007).

A layer of litter (3.5 g, mixed litter material collected at the field site of the B4WarmED experiment at Cloquet;

dried at 60°C for three days and cut into pieces about 3 cm in length) was placed on the soil surface to simulate natural conditions. The leaf litter material consisted mainly of *Populus tremuloides*, *Aster macrophyllus* and *Carex pensylvanica*. Then, we added 15 seeds each of four native and four exotic plant species (120 seeds microcosm^{-1}; ~15 300 seeds m^{-2}), which is in line with previous seed addition experiments (Myers and Harms 2009). Native and exotic species were chosen from three functional groups, forbs (broadleaved herbs), legumes(Fabaceae), and grasses (Poaceae). As native plant species we used *A. macrophyllus* (forb), *Aquilegia canadensis* (forb), *Calamagrostis canadensis* (grass) and *Lathyrus venosus* (legume); as exotic plant species we used *Plantago major* (forb), *Taraxacum officinale* (forb), *Phleum pratense* (grass) and *Trifolium pratense* (legume) (see Supplementary material Appendix 1, Table A1 for more details). All eight plant species are common at the Cloquet field site. The native plant species were purchased from Prairie Moon Nursery (MN, USA), the exotic species from Rieger-Hofmann GmbH (Germany). Seed dry weight (as a proxy measure for seed size) did not differ significantly between native and exotic species (F$_{1,6}$ = 0.48, p = 0.51) although different species varied considerably (Supplementary material Appendix 1, Table A1).

Earthworms (*A. caliginosa*, *L. rubellus* and *Lumbricus terrestris*) were purchased from Blue Ribbon Bait and Tackle Shop (MN, USA) and kept in the experimental soil for two weeks. Then, we added either no earthworms (control), two adult individuals of *A. caliginosa* ($\sum$ 1.43 $\pm$ 0.01 g fresh weight with gut content), two adult individuals of *L. rubellus* ($\sum$ 1.13 $\pm$ 0.01 g), one adult individual of *L. terrestris* (4.66 $\pm$ 0.13 g) or the same number of all three species, i.e. two *A. caliginosa*, two *L. rubellus* and one *L. terrestris*, to the microcosms to create five earthworm treatments with 12 replicates each. Half of the replicates were incubated at ambient temperature (two replicates in each of three growth chambers), the other half at +4°C (5 earthworm treatments × 2 temperature treatments × 6 replicates = 60 microcosms). It should be noted that earthworm treatments were confounded with earthworm biomass because treatments containing *L. terrestris* had considerably higher earthworm biomass than treatments with *A. caliginosa* and *L. rubellus* only. We chose this approach to simulate natural earthworm abundances at the B4WarmED experiment at Cloquet.

The experiment lasted 36 days. This duration was sufficient to cover the main treatment effects on litter removal and germination of seedlings as indicated by saturating relationships between processes and time (Supplementary material Appendix 1, Fig. A1). Light intensity varied between 580 and 900 µE m^{-2} s^{-1} depending on the location in the growth chambers. To avoid chamber edge effects, microcosms were randomized every week within each chamber. The microcosms were irrigated four times per week with deionized water and the volume was increased from 50 ml (weeks 1–3) to 100 ml (weeks 4 and 5). Therefore, all microcosms received the same amount of water allowing for treatment-induced differences in water availability, e.g. due to higher evapotranspiration and/or preferential flow of soil surface water in earthworm burrows (Shipitalo et al. 2004) and drainage through the bottom of the microcosms.

Sampling

We identified and counted the number of germinated seedlings 8, 15, 22, 29 and 36 days after adding the seeds. On the same days we estimated the coverage of the litter layer (% coverage of soil surface) to assess litter removal (as proxy measure for litter decomposition) as one crucial ecosystem process and factor potentially affecting seedling establishment. On day 36 we stopped the experiment and harvested the shoot material of each species separately by cutting shoots at the soil surface level. Then, we collected the remaining litter material. Shoot and litter material were dried at 70°C for three days. Litter dry mass at the end of the experiment correlated well with litter coverage (R^2 = 0.81, p < 0.0001), allowing us to use 100%-coverage (%) as proxy measure for litter removal for all five measurements in time. Total seedling number did not correlate with total seedling biomass (R^2 < 0.01, p = 0.95), prompting us to analyze both seedling performance properties separately. In addition, we calculated the mean biomass per plant individual. We grouped native and exotic species to investigate if warming and earthworm presence influenced their relative abundance. We differentiated native and exotic seedling numbers only at the end of the experiment due to initially low seedling numbers of native species.

We broke up the soil core and took samples (~30 g soil fresh weight) from the upper 5 cm and the lower 5 cm of the soil column to investigate treatment effects on soil water content at different soil depths. Soil samples were dried (70°C for three days) and the gravimetric soil water content (%) was determined as the weight difference of fresh and dried soil. Earthworms were collected by hand, weighed individually (fresh weight with gut content) to investigate the effect of warming and co-occurrence with other earthworm species on earthworm performance (change of earthworm weight). Soil columns were inspected qualitatively for earthworm activity (number of earthworm burrows, coverage of litter layer) to verify that earthworms had been active during the experiment. One microcosm (*L. terrestris* treatment at ambient temperature) had to be excluded from the analyses because we did not recover the added earthworm and there were no signs of earthworm activity.

Statistical analyses

Data on seedling number and biomass were log-transformed and percentage data were arc-sin-transformed to meet the requirements of analysis of variance (ANOVA; normality and homoscedasticity of errors), if necessary. Means ($\pm$SE) presented in text and figures were calculated using non-transformed data. ANOVA was performed to analyze the effects of temperature (TE; ambient and +4°C), earthworms (EW; control, *A. caliginosa*, *L. rubellus*, *L. terrestris*, all three species) and TE × EW on soil water content (top and bottom), litter removal, total number of seedlings (microcosm^{-1}), number of native seedlings, number of exotic seedlings, total seedling biomass, biomass of native seedlings, biomass of exotic seedlings, total seedling species richness, native species richness, exotic species richness, biomass per plant individual (total), biomass per native plant individual, biomass per exotic plant individual, ratio

between the number of exotics to natives, ratio between the diversity of exotics to natives, and ratio between the biomass of exotics to natives (all at the end of the experiment). The effect of TE was tested against the random effect of chamber nested within TE. In addition, we performed repeated measures ANOVA to analyze the effects of time (TI; day 8, 15, 22, 29, 36), TE, EW and all possible two and three way interactions on litter removal, total seedling number and total seedling species richness. Significant three way interactions allowed us to perform separate ANOVAs for each measurement in time. Moreover, ANOVA was performed to analyze the effect of TE and EW (monoculture and three species mixture) on the weight change (performance) of *A. caliginosa*, *L. rubellus* and *L. terrestris*. Comparison of means were performed using Tukey's HSD test ($\alpha = 0.05$). All analyses were performed using STATISTICA 7 (Statsoft).

In order to investigate treatment effects on plant community composition, we used two complimentary nonparametric multivariate techniques, non-metric multidimensional scaling (NMDS) and multi-response permutation procedures (MRPP). Analyses were carried out using the R 'vegan' package (ver. 1.17-6) (R Development Core Team 2008, Oksanen et al. 2011). NMDS, an unconstrained ordination method robust for ecological data, is based on ranked distances among sample units and uses an iterative search to depict these data in as few dimensions as possible while minimizing stress (Minchin 1987, McCune and Grace 2002). A two-dimensional solution was sought using the defaults of the metaMDS function. Species scores along the ordination axes were calculated based on weighted averages of the sample units (Oksanen et al. 2011). For interpretation, the axes were rigidly rotated to place the strongest correlation between species composition and an environmental variable on the first axis. EW and TE treatments as well as soil water and litter biomass vectors were fit to the ordination using the envfit function which provides a goodness of fit and p-value based on permutation tests. MRPP was then run to test for pairwise differences in treatment group composition by comparing dissimilarities within and among treatment groups with random samples of plots. MRPP provides two pieces of information, the agreement statistic 'A' which is a measure of within group agreement and a p-value. MRPP and NMDS operate on dissimilarity matrices and we utilized the asymmetrical Bray-Curtis distance measure, which performs well with plant community data (Legendre and Legendre 1998, McCune and Grace 2002). Plant species stem density values were initially relativized by species maximum to create a more equal weighting of common and rare species (McCune and Grace 2002). We did not correct for multiple statistical tests considering the mathematical and logical argumentation by Moran (2003).

In addition to ANOVA, we used structural equation modeling (SEM) to investigate how warming and different earthworm ecological groups directly and indirectly affect the establishment of exotic and native plants. SEM allows testing direct and indirect relationships between variables in a multivariate approach (Grace 2006). The initial model was based on previous knowledge (Supplementary material Appendix 1, Fig. A3) testing direct effects of earthworms and warming (exogenous variables) on seedling performance (endogenous

variables: number, biomass and species richness). Moreover, we tested indirect relationships of exogenous variables via soil water content and litter biomass (endogenous variables). The adequacy of the model was determined via χ^2-tests, AIC and RMSEA. Adequate model fits are indicated by a nonsignificant χ^2-tests (p > 0.05), low AIC and low RMSEA (<0.05) (Grace 2006, Arbuckle 2010). According to the ANOVA approach, three separate models were determined for seedling number, seedling biomass and seedling species richness. Due to the complete factorial design and nonsignificant effects of our treatments on earthworm performance we expected exogenous variables to be independent from each other. Model modification indices and stepwise removal of unimportant relationships were used to improve the models (based on the model fit indices mentioned above); however, only scientifically sound relationships were considered (Grace 2006). Standardized path coefficients are given in Fig. 5, while unstandardized path coefficients are given in Fig. A3. SEM was performed using Amos 5 (Amos Development Corporation, Crawfordville, FL, USA).

Results

Earthworms

Both earthworm survival and body weight change were unaffected by our treatments (all p > 0.26; Supplementary material Appendix 1, Table A2). Overall, 88% of the *A. caliginosa*, 57% of the *L. rubellus* and 96% of the *L. terrestris* individuals were recovered from the microcosms at the end of the experiment. *A. caliginosa* (−11%) and *L. rubellus* (−51%) lost body weight during the experiment, whereas the body fresh weight of *L. terrestris* did not change during the experiment (+ 1%). All *L. rubellus* microcosms showed high earthworm activity (litter removal from the soil surface and number of earthworm burrows; not shown), suggesting that *L. rubellus* individuals escaped towards the end of the experiment due to low litter availability. These microcosms were included in the analyses.

Soil water content

Treatment effects on gravimetric soil water content were significant for the upper soil layer (overall mean ± SE: 15.7 ± 0.5%), whereas the lower soil layer remained unaffected (10.6 ± 1.0%; Supplementary material Appendix 1, Table A3). Lower soil water content at higher temperature resulted from a significant interaction between temperature and earthworms ($F_{4,44} = 2.65$, p = 0.046); while top soil water content was virtually unaffected by temperature in the absence of earthworms (−3%), it was considerably lower in the warmed treatment in the presence of *A. caliginosa* (−24%), *L. rubellus* (−19%), *L. terrestris* (−37%), and all three earthworm species (−31%; Fig. 1a).

Litter removal

Litter mass was affected by a significant three way interaction between temperature, earthworms and time ($F_{4,45} = 3.32$, p = 0.018; Supplementary material Appendix 1, Table A4).
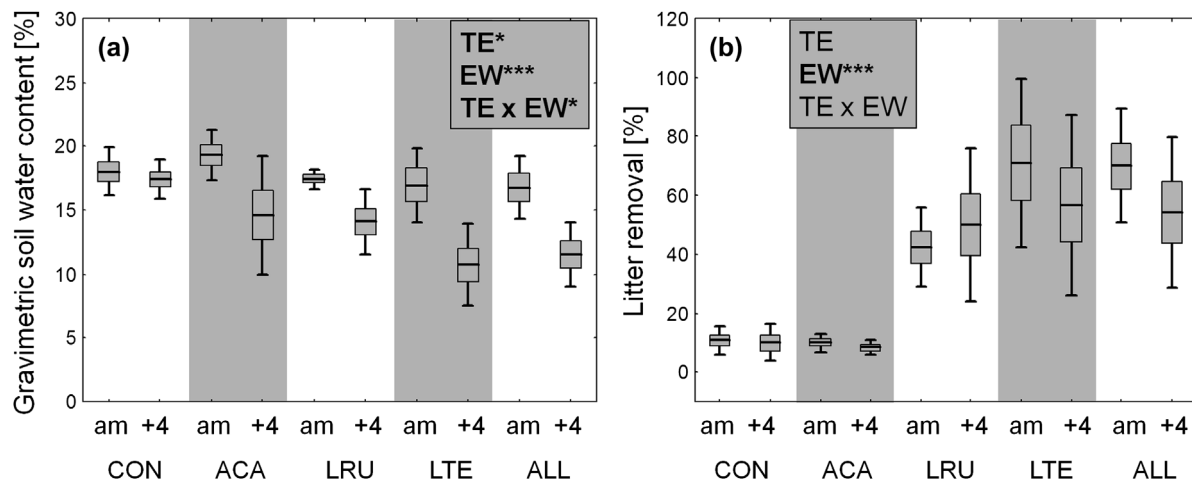
Figure 1. Gravimetric soil water content in the upper 5 cm (a) and litter removal (b) as affected by temperature (TE; am = ambient, $+4 = +4°C$) and earthworms (EW; CON = control without earthworms, ACA = *Aporrectodea caliginosa* (endogeic), LRU = *Lumbricus rubellus* (epi-endogeic), LTE = *Lumbricus terrestris* (anecic), ALL = combination of all three earthworm species) at the end of the experiment. Means with standard error and standard deviation. * = $p < 0.05$, *** = $p < 0.001$ (Supplementary material Appendix, Table A3).

While litter removal was low in the control and the *A. caliginosa* treatments (Fig. 1b, Supplementary material Appendix 1, Fig. A2a), it increased over time in the presence of *L. rubellus*, *L. terrestris* and all three earthworm species, respectively (Fig. 1b). Litter removal was higher at ambient temperature than at $+4°C$ in the presence of *L. terrestris* and all three earthworm species, but did not change with temperature in the other treatments (Fig. 1b, Supplementary material Appendix 1, Fig. A2a).

Seedling number

Seven plant species germinated during the experiment; *A. macrophyllus* did not germinate, neither in the main experiment nor in an additional seed germination experiment (not shown). Thus, the results shown below refer to three native and four exotic plant species (Supplementary material Appendix 1, Table A1); with exotics being both more numerous and larger on average than the natives across all treatments. The total number of seedlings was affected by a significant three way interaction between temperature, earthworms and time ($F_{4,45} = 2.97$, $p = 0.029$; Supplementary material Appendix 1, Table A4). The number of seedlings increased over time, and this was most pronounced in the absence of earthworms (Fig. 2a, Supplementary material Appendix 1, Fig. A2b).

In general, significantly more native seedlings germinated in the warmed in comparison to the ambient treatment (Supplementary material Appendix 1, Table A3, Fig. 2b), although not in *L. rubellus* treatments (Fig. 2b). In contrast to native species, seedling numbers of exotic plant species were little affected by temperature but varied considerably between the earthworm treatments (Supplementary material Appendix 1, Table A3, Fig. 2c). Exotic seedling numbers were significantly lower in *L. terrestris* treatments and treatments with all three earthworm species than in the control and in the presence of *A. caliginosa* (Fig. 2c). Moreover, exotic seedling numbers were significantly lower in *L. rubellus*

treatments than in control treatment, but significantly higher than in *L. terrestris* treatments and all three earthworm species (Fig. 2c).

Seedling biomass

Total seedling biomass per microcosm differed significantly between earthworm treatments ($F_{4,44} = 6.56$, $p < 0.001$), whereas temperature had no significant effect ($F_{1,4} = 2.67$, $p = 0.18$; Supplementary material Appendix 1, Table A3). Seedling biomass was significantly higher in the presence of all three earthworm species and *L. rubellus* only than in controls and the treatment with *L. terrestris* only (Fig. 2d). The differences in total seedling biomass could be ascribed to changes in the biomass of exotic plant species, because the biomass of native seedlings remained little affected (Supplementary material Appendix 1, Table A3, Fig. 2e–f).

The biomass per seedling individual was significantly higher for exotics and all plants pooled in the presence of all three earthworm species than the control and the sole presence of *A. caliginosa* or *L. terrestris* (Supplementary material Appendix 1, Table A3, Fig. 2g, 2i). This effect was only true for exotic plant species as natives were not significantly affected by treatments (Fig. 2h–2i).

Seedling species richness

Seedling species richness was significantly affected by earthworms ($F_{4,44} = 6.19$, $p < 0.001$), whereas temperature had no significant effect ($F_{1,4} = 0.42$, $p = 0.55$; Supplementary material Appendix 1, Table A3, Fig. 2j). Seedling species richness at the end of the experiment was significantly higher in the control than in the sole presence of *L. rubellus* or *L. terrestris* as well as the presence of all three earthworm species (Fig. 2j). Moreover, seedling species richness was significantly higher in the sole presence of *A. caliginosa* than in the presence of all three earthworm species (Fig. 2j).
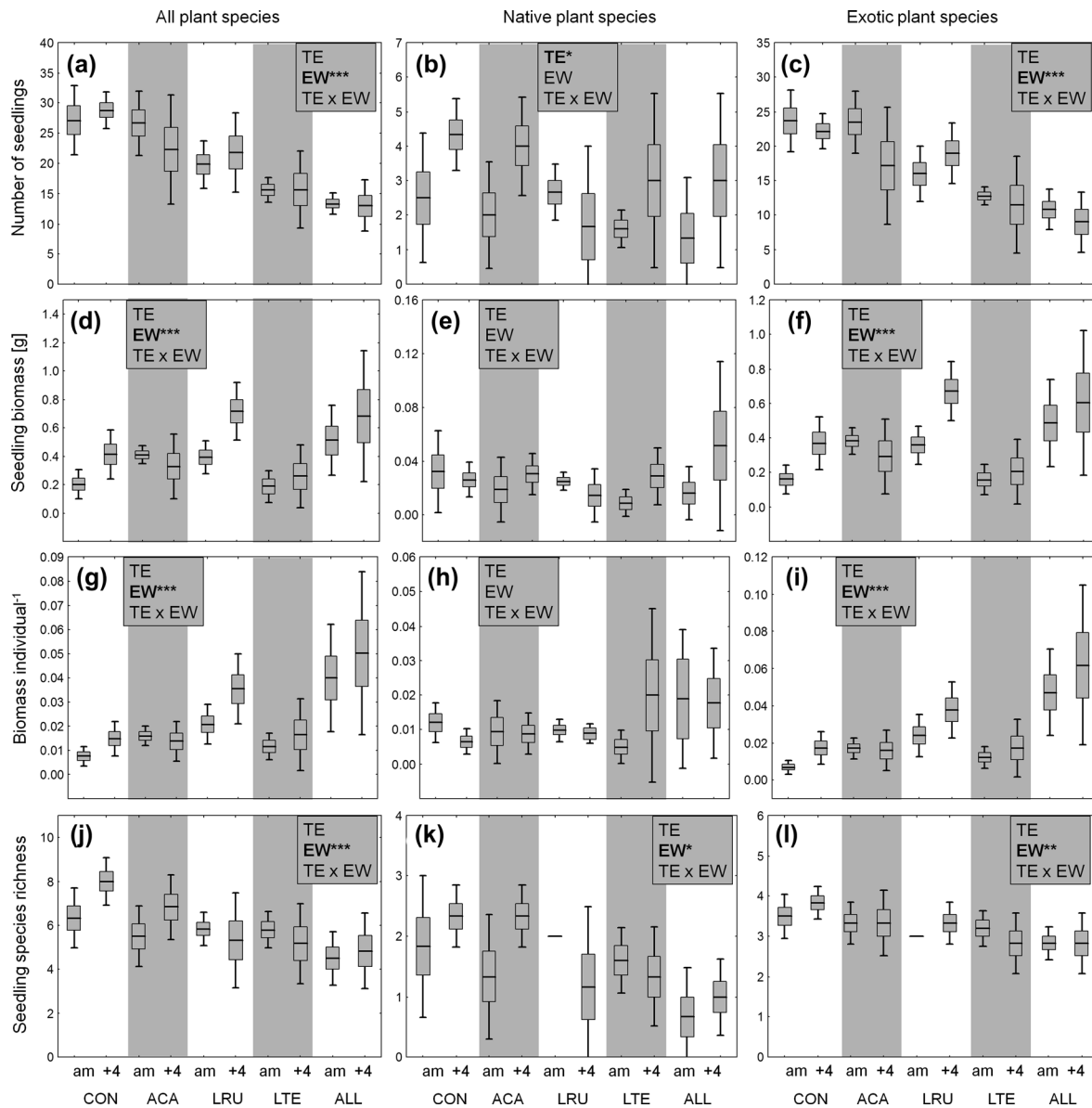
Figure 2. Number (a, b, c), biomass (d, e, f), individual biomass (g, h, i), and species richness (j, k, l) of total (a, d, g, j), native (b, e, h, k), and exotic seedlings (c, f, i, l) as affected by temperature (TE; am = ambient, + 4 = + 4°C) and earthworms (EW; CON = control without earthworms, ACA = *Aporrectodea caliginosa* (endogeic), LRU = *Lumbricus rubellus* (epi-endogeic), LTE = *Lumbricus terrestris* (anecic), ALL = combination of all three earthworm species) at the end of the experiment. Means with standard error and standard deviation. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 (Supplementary material Appendix, Table A3).

These patterns were similar for exotic and native species viewed separately.

Seedling species richness was significantly affected by a three way interaction between time, temperature and earthworms ($F_{4,45} = 4.06$, p = 0.007; Supplementary material Appendix 1, Table A4, Fig. A2c). Seedling species richness generally increased over time, although this was most pronounced in controls at elevated temperature (Supplementary material Appendix 1, Fig. A2c).

Ratio between exotic and native plant species

While the ratio between the biomass of exotic versus native plant species was not significantly affected by our treatments

(all p > 0.29), the ratios between the number as well as the species richness of exotic versus native plant species were significantly affected by the interaction between temperature and earthworms ($F_{4,44} = 3.65$, p = 0.012 and $F_{4,44} = 2.62$, p = 0.047, respectively; Supplementary material Appendix 1, Table A3). The ratio between the number of exotic versus native plant species was lower in the warmed than in the ambient treatment in the control and the sole presence of *A. caliginosa*, whereas the opposite was true in the sole presence of *L. rubellus* (Fig. 3a). Similarly, the ratio between the species richness of exotic versus native plant species was lower in the warmed than in the ambient treatment in the sole presence of *A. caliginosa*, whereas it was higher in the sole presence of *L. rubellus* (Fig. 3b).
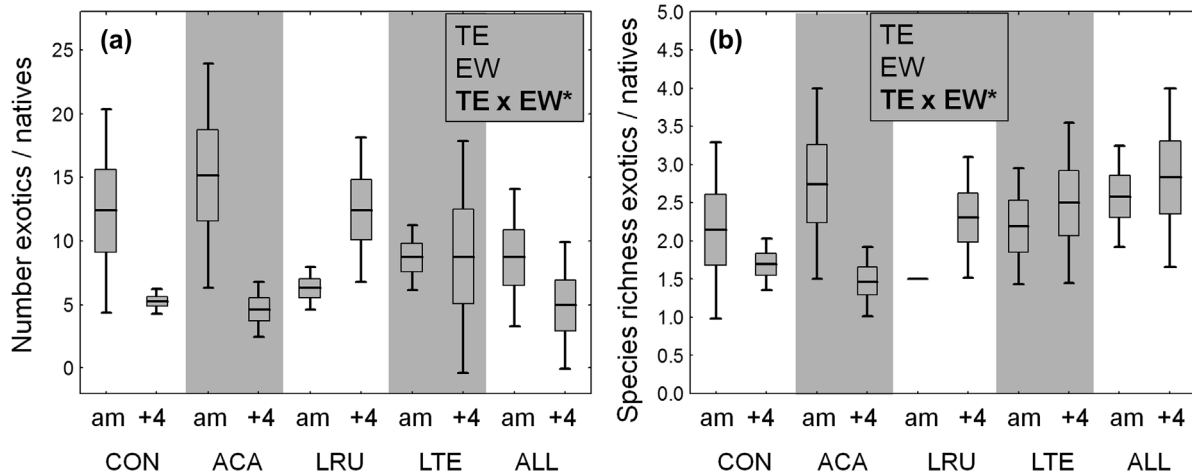
Figure 3. The ratio between the number (a) and the species richness (b) of exotic to native seedlings as affected by temperature (TE; am = ambient, + 4 = + 4°C) and earthworms (EW; CON = control without earthworms, ACA = *Aporrectodea caliginosa* (endogeic), LRU = *Lumbricus rubellus* (epi-endogeic), LTE = *Lumbricus terrestris* (anecic), ALL = combination of all three earthworm species) at the end of the experiment. Means with standard error and standard deviation. * = p < 0.05 (Supplementary material Appendix, Table A3).

Plant community composition

NMDS ordination of plant community composition showed greater differences by earthworm than temperature treatments (Fig. 4). Plant community centroids for earthworm and temperature treatments, and soil water and litter biomass vectors are shown along with the species centroids in Fig. 4. The two-dimensional solution had a final stress of 16.78, RMSE of 0.0017, and non-metric fit R^2 of 0.96, indicating a satisfactory fit and stable solution. Both soil water and litter biomass had significant goodness of fit statistics ($R^2 = 0.27$, p < 0.001 and $R^2 = 0.12$, p = 0.03, respectively) and showed increasing values along axis 1. Earthworm treatment centroids also had significant goodness of fit ($R^2 = 0.29$, p < 0.001) and separated into two groups along axis 1, *L. terrestris* and all three species treatments to the left of ordination center and the *L. rubellus*, *A. caliginosa*, and control treatments on the right side. Pairwise MRPP tests indicated homogeneity within and significant plant community differences among these two groupings of earthworm treatments (Table 1). The *L. terrestris* and all three species treatments did not differ significantly in plant composition, and the *L. rubellus*, *A. caliginosa*, and control treatments also did not differ from one another (Table 1). All pairwise comparisons among these two groups were significant, with the strongest compositional difference being between the control and all three species treatments (Table 1). Temperature treatments differentiated from one another along axis 2 although the goodness of fit was only marginally significant ($R^2 = 0.05$, p = 0.07) and the MRPP test did not find significant compositional differences (A = 0.019, p = 0.494).

The community level responses were driven by strong differences between native and exotic species. The centroids of the four exotic species, *T. officinale*, *P. pratense*, *P. major* and *T. pratense* were found on the right hand side of axis 1, indicating relatively higher abundances in the *L. rubellus*, *A. caliginosa* and control treatments. The three native species, *L. venosus*, *A. canadensis* and *C. canadensis* exhibited more

variable responses. *Aquilegia canadensis* density was greatest in the control and *A. caliginosa* treatments while *L. venosus* and *C. canadensis* differentiated from the other plant species along axis 2, indicating that their densities were not driven by EW treatments or responses to soil water and litter.
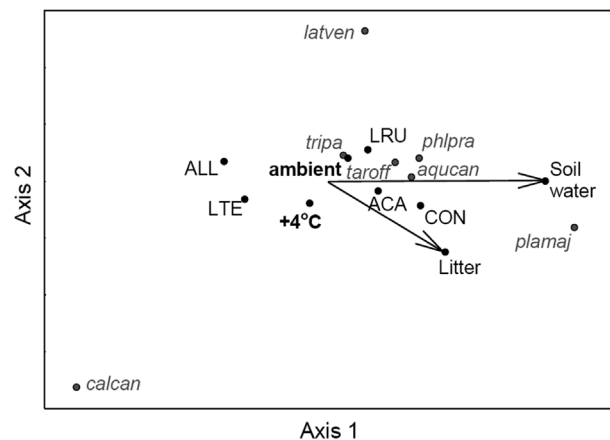

Figure 4. Non-metric multidimensional scaling results on the effects of temperature (ambient, + 4°C), earthworms (CON = control without earthworms, ACA = *Aporrectodea caliginosa* (endogeic), LRU = *Lumbricus rubellus* (epi-endogeic), LTE = *Lumbricus terrestris* (anecic), ALL = combination of all three earthworm species) and soil water content and aboveground litter biomass on the composition of the plant community (density of plant individuals per species). Continuous factors (soil water content and litter biomass) are represented by vectors, whereas categorical factors (earthworm treatments and warming treatments) are represented by their compositional centroids, or mean location in species space. Species centroids indicate their mean location or 'center of gravity'. *aqucan* = *Aquilegia canadensis* (native forb), *calcan* = *Calamagrostis canadensis* (native grass), *latven* = *Lathyrus venosus* (native legume), *plamaj* = *Plantago major* (exotic forb), *taroff* = *Taraxacum officinale* (exotic forb), *phlpra* = *Phleum pratense* (exotic grass) and *tripra* = *Trifolium pratense* (exotic legume). Plant species are given in grey, earthworm treatments in capitals and temperature treatments in bold. Vectors are highlighted with arrows.

Table 1. MRPP (multi-response permutation procedure) results of the comparison of plant community composition (seedling density per plant species) as effected by earthworm treatments (EW; CON = control without earthworms, ACA = *Aporrectodea caliginosa* (endogeic), LRU = *Lumbricus rubellus* (epi-endogeic), LTE = *Lumbricus terrestris* (anecic), ALL = combination of all three earthworm species). Given are the pairwise comparisons between treatments. Correcting for multiple tests would have rendered the comparisons between ACA vs LTE, LRU vs LTE and LRU vs ALL insignificant (but see Moran 2003). A = within group agreement. Significant effects (p < 0.05) are given in bold.

Overall			A	p-value
Pairwise comparisons			0.093	0.002
EW		EW	A	p-value
CON	vs	ACA	−0.016	0.686
CON	vs	LRU	0.056	0.061
CON	vs	LTE	0.099	**0.003**
CON	vs	ALL	0.173	**0.001**
ACA	vs	LRU	0.033	0.129
ACA	vs	LTE	0.052	**0.045**
ACA	vs	ALL	0.123	**0.003**
LRU	vs	LTE	0.039	**0.039**
LRU	vs	ALL	0.065	**0.038**
LTE	vs	ALL	−0.018	0.659

Exploring the mechanisms – structural equation modeling

The SEMs supported the results of the ANOVA and NMDS approaches, but, in addition, helped to identify possible mechanisms. Generally, we were able to predict the performance of exotic plants better than that of native plants (Fig. 5), which is not surprising given that native plants showed inconsistent responses to our treatments (Fig. 4).

The initial model (Supplementary material Appendix 1, Fig. A3) for seedling number fit the data well ($\chi^2_{11} = 7.19$, p = 0.78; AIC = 57.19), but could be improved (Fig. 5a, A3b; $\chi^2_{10} = 4.98$, p = 0.89; AIC = 40.98). *Lumbricus terrestris* and *L. rubellus* had direct detrimental effects on the number of exotic seedlings, but positive indirect effects *via* removal of the litter layer. Moreover, *L. terrestris* indirectly, detrimentally affected the number of exotic plants by decreasing soil water content. The number of native seedlings increased marginally with warming and indirectly due to reduced soil water content in the warmed treatment. Moreover, earthworms exerted an indirect negative effect on the number of native plants by removing the litter layer.

The initial model for seedling biomass did not fit the data well ($\chi^2_{11} = 22.04$, p = 0.02; AIC = 72.04), and was improved as illustrated in Fig. 5b (Supplementary material Appendix 1, Fig. A3c; $\chi^2_{15} = 7.80$, p = 0.93; AIC = 49.80). The biomass of exotic plants was directly positively affected by warming and the presence of *A. caliginosa* and *L. rubellus*. Moreover, *L. terrestris* and *L. rubellus* had an indirect positive effect by reducing the litter layer. However, *L. terrestris* had a marginal direct negative effect on the biomass of exotics, as well as an indirect negative one by decreasing soil water content. The biomass of native plants was only marginally affected by warming and the presence of *A. caliginosa*.

The initial model for species richness of seedlings fit the data well ($\chi^2_{11} = 11.16$, p = 0.43; AIC = 61.16), but could be improved (Fig. 5c, Supplementary material Appendix 1, Fig. A3d; $\chi^2_8 = 3.53$, p = 0.90; AIC = 43.53). The presence of *L. terrestris* and *L. rubellus* directly detrimentally affected the species richness of exotic and native seedlings. Moreover, *L. terrestris* had an indirect negative effect on the species richness of exotics by decreasing soil water content. In addition to negative effects of earthworms on exotic species richness, they had an indirect positive effect through decreasing the litter layer. Warming had a direct positive effect on species richness of exotic seedlings but an indirect negative effect by decreasing soil water content. The species richness of native seedlings was negatively affected by *L. terrestris* and *L. rubellus*, but benefitted indirectly from the presence of *L. terrestris* and warming through decreased soil water content.

Discussion

Hypothesis 1: anecic and epigeic earthworms affect seedling establishment

In accordance with our hypothesis, anecic and epigeic earthworms had the strongest effect on seedling establishment. While the richness and the number of exotic seedlings decreased in the presence of these earthworm ecological groups, the biomass per microcosm and per plant individual increased, confirming the results of previous studies (Milcu et al. 2006, Eisenhauer and Scheu 2008). SEM helped to address underlying mechanisms: anecic and epigeic earthworms had direct detrimental effects on seedling number and richness, suggesting that they buried and/or fed on seeds and seedlings (Fig. 5; Regnier et al. 2008, Eisenhauer et al. 2010). In addition, both earthworm ecological groups affected seedling establishment via decreasing soil water content and surface litter biomass. In contrast to the mostly detrimental effects on seedling number and species richness, earthworm effects on seedling biomass were largely positive, which was likely due to enhanced nutrient availability (Scheu 2003) and/or reduced competition.

Anecic earthworms are known to play a crucial role in seed dispersal, burial and seedling establishment (Milcu et al. 2006, Eisenhauer et al. 2008b, Regnier et al. 2008). The underlying mechanisms are multifaceted (Forey et al. 2011), though detrimental effects prevailed in our experiment. Those include transporting seeds to a critical depth in soil where they fail to emerge (Traba et al. 1998, Regnier et al. 2008), damaging or digesting seeds during earthworm gut passage (McRill and Sagar 1973, Shumway and Koide 1994) as well as seed and seedling predation (Asshoff et al. 2010, Eisenhauer et al. 2010). The NMDS and SEM results indicate that reduced soil water content as well as the removal of the litter layer partly explain the reduced number and species richness of seedlings in the treatments with anecic and epigeic earthworms. Earthworm burrows, particularly the vertical ones formed by anecic species, are known to function as preferential flow pathways for soil surface water (Edwards and Bohlen 1996, Shipitalo et al. 2004). However, once established, seedlings may have benefited from reduced competition and better nutrient availability resulting in increased seedling biomass (Milcu et al. 2006, Eisenhauer and Scheu 2008), particularly in the presence of epigeic
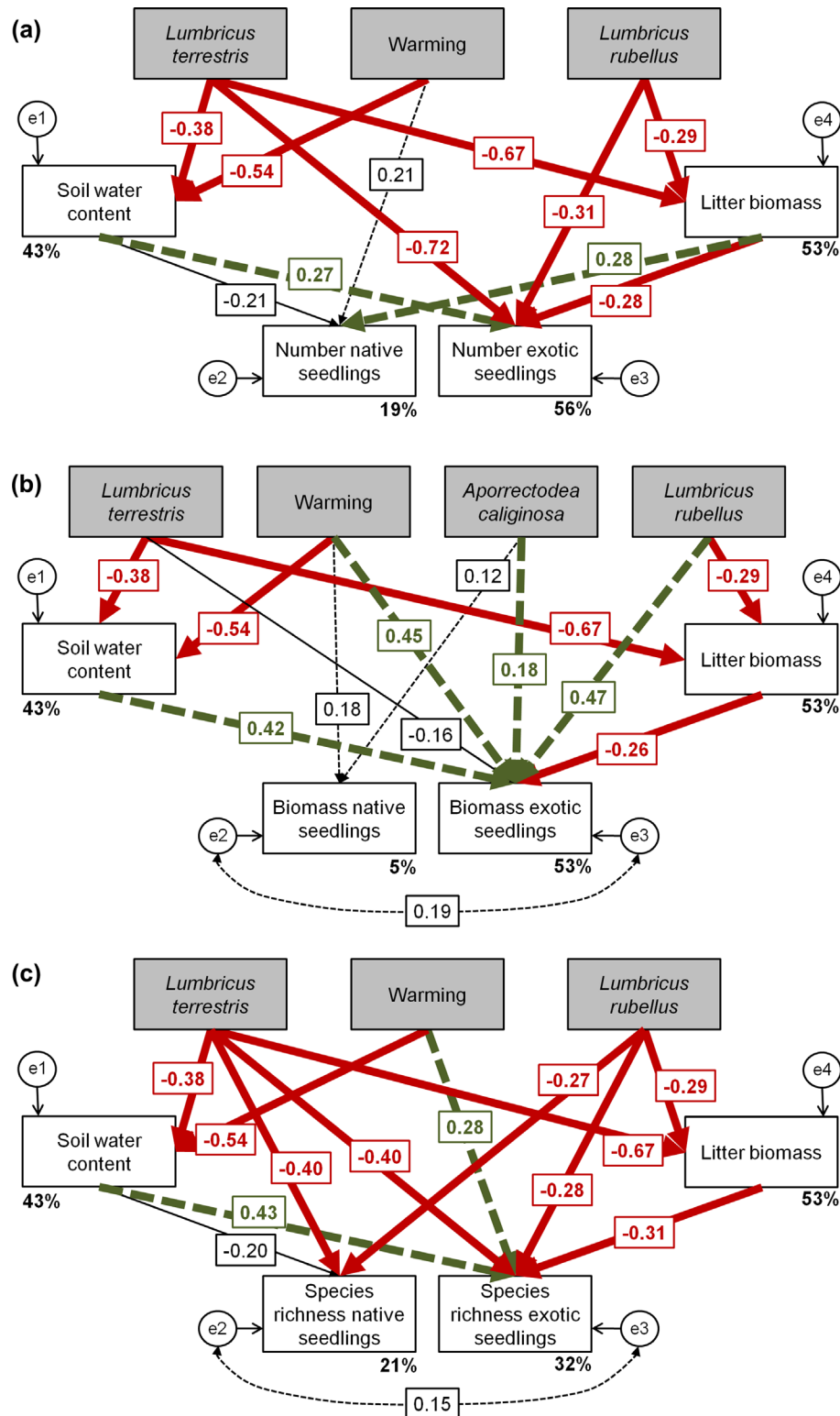
Figure 5. Structural equation models (SEM) of causal influences of presence of different earthworm species and warming (exogenous variables; grey rectangles) on soil water content, litter biomass and performance (number, biomass or species richness) of exotic and native seedlings (endogenous variables; white rectangles) on (a) seedling number, (b) seedling biomass and (c) seedling species richness. Numbers on arrows are standardized path coefficients (equivalent to correlation coefficients). All final models fit the data well (see text for details). Width of the arrows indicated the strength of the causal influence: bold arrows indicate significant standardized path coefficients (p < 0.05), fine arrows indicate non-significant path coefficients (p > 0.05), double-headed arrows indicate correlations between error terms. Black arrows indicate non-significant relationships, green dashed arrows significant positive relationships and red solid arrows significant negative relationships. Circles indicate error terms (e1–e4). For more information see main text, Supplementary material Appendix, Table A5 and Fig. A3.

and endogeic earthworms (Fig. 5b). The endogeic species *A. caliginosa* had little effect on seedling number and richness, but increased seedling biomass. Endogeic earthworms are known to increase soil nutrient availability (Scheu 2003, Partsch et al. 2006) and this may have played a role in well-established plants with a more pronounced root system.

Our findings show that, in addition to direct interactions between earthworms and seeds via seed predation, earthworms induce a suite of ecosystem changes, i.e. changes in soil water content, litter cover and nutrient availability, affecting this bottleneck stage of plant regeneration. Although the varying earthworm treatments were confounded with earthworm biomass, this is likely to realistically reflect natural conditions.

Hypothesis 2: warming decreases the number and biomass of seedlings

Overall, the main effect of warming on the total number, biomass and species richness of established seedlings was not significant in the ANOVA approach. However, and contradicting our hypothesis, the number of native seedlings increased significantly in the warmed treatment. As indicated by the NMDS results, native plant species differed considerably in their response to warming: the grass *C. canadensis* had higher numbers of seedlings in the warmed treatment, while the legume *L. venosus* showed the opposite pattern. The SEM results suggest that warming affected seedling establishment indirectly *via* reducing soil water content (Fig. 5). In addition, once established, exotic species built more biomass in the warmed treatment. Previous studies showed mixed effects of warming on seedling establishment: both positive (Milbau et al. 2009) and negative impacts (Shevtsova et al. 2009) have been reported in subarctic regions, and little (Lloret et al. 2009) to negative effects (Lloret et al. 2005) have been documented in Mediterranean shrubland. Thus, impacts of warming on seedling establishment may be plant species specific (Shevtsova et al. 2009) and context dependent. In line with our findings, Kimball et al. (2010) concluded that detailed ecophysiological knowledge of individual species is necessary to understand complex shifts in plant community composition in response to global change. Moreover, Shevtsova et al. (2009) found temporal and species specific variation in the sensitivity of seedling establishment to warming. Although our experiment did not allow the investigation of seasonal variability in warming effects, the present results underline plant species specific responses. Warming had little effects on earthworm activity and litter removal, but it is likely to select for plant species being able to tolerate water stress during seedling establishment, particularly in the presence of anecic and epigeic earthworms.

Hypothesis 3: warming and earthworms interactively affect seedling establishment

Assuming the existence of some distinct non-additive mechanisms underlying the effects of warming and worming on seedling establishment, we hypothesized interactive effects of these environmental change agents. Indeed,

confirming our expectations, we found significant interactions on soil water content (of the upper 5 cm), litter removal, and seedling establishment over time. It is well-known that warming often results in lower soil water content due to increased evapotranspiration (Bontti et al. 2009, Kardol et al. 2011). However, our results show that warming had only little effect in the control treatment, but soil water content was significantly reduced in warmed treatments in the presence of earthworms. Four not mutually exclusive mechanisms may have caused this interaction, probably acting in concert. First, plants built more biomass in some of the earthworm treatments, hence increasing plant transpiration. This was supported by a negative (albeit weak) correlation between total plant biomass per microcosm and soil water content ($R^2 = 0.07$, p = 0.040). Second, anecic and epigeic earthworms reduced the litter layer considerably, most likely resulting in higher evaporation. We found however no support for this assumption ($R^2 = 0.01$, p = 0.54; Fig. 5). Third, earthworm burrows function as preferential flow pathways for soil surface water (Edwards and Bohlen 1996, Shipitalo et al. 2004), facilitating water transport into deeper soil layers, i.e. drainage of water at the bottom of the microcosms in the present study, and reducing soil water content in upper soil layers (Fig. 5). Fourth, earthworms are known to change soil structure by e.g. soil compaction (Edwards and Bohlen 1996, Eisenhauer 2010), thereby potentially reducing soil water holding capacity. As discussed above, this may have detrimentally affected seedling number and species richness.

Litter removal was predominantly driven by anecic and epigeic earthworms. However, we found a significant interaction between earthworm treatment, warming and time; litter removal only increased in the ambient treatment in the presence of anecic and all earthworms towards the end of the experiment. Although warming did not affect earthworm performance significantly, higher temperatures may have decreased the litter burial activity of anecic earthworms towards the end of the experiment. Frelich and Reich (2010) posited that global warming will reinforce the spread and impacts of invasive earthworms in northern North America. However, this may be constrained by warming-related soil drying. Zaller et al. (2009) found that moderate experimental warming significantly reduced the density and biomass of epigeic earthworms in a *Carex* fen ecosystem in southern South America. This topic certainly deserves further attention. Given the pivotal role of the litter layer for the germination of many herbaceous and tree species in forest ecosystems (Frelich et al. 2006), it is likely that interactive effects of warming and worming will have strong effects on plant regeneration.

The SEM results suggest that the invasion of anecic earthworms and warming are likely to have synergistic effects on seedling establishment as they decrease soil water content and thus increase water stress for germinating plants if operating in concert. Moreover, the removal of the insulating litter layer by anecic and epigeic earthworms is likely to amplify water stress during summer. Thus, warming and worming are likely to strongly decrease herbal layer species richness by favouring drought-tolerant species which survive direct interactions with earthworms.

Hypothesis 4: warming and earthworms shift plant composition towards exotic species

We expected that warming and earthworms would shift the plant community towards the exotic species present because they may be better adapted to the presence of earthworms (Forey et al. 2011) and higher temperatures. Conversely, we found that warming mostly benefited native plant species. This effect was even more pronounced in the presence of endogeic earthworms, which most likely did not directly interact with seeds (Asshoff et al. 2010) but increased nutrient availability (Scheu 2003, Wurst et al. 2005). However, it should be noted that the native grass species *C. canadensis* benefited from warming-induced reduced soil water content. In contrast, anecic earthworms counteracted the beneficial effect of warming on native plants, and epigeic earthworms even reversed the effect with higher abundance and richness ratios of exotics and natives in the warmed treatment.

The SEM results indicate that three main mechanisms changed the ratio between exotic and native plant species. First, exotic plant species benefitted from the earthworm-induced decrease in litter cover, whereas native plant species germinated better in the presence of a pronounced litter layer. This finding has important implications as many native forest plant species may need a pronounced litter layer to germinate (Frelich et al. 2006). Second, reduced soil water content in warmed treatments and in the presence of anecic earthworms detrimentally affected exotic plants, while native plants benefited marginally. Third, seed and seedling predation detrimentally affected both the number and species richness of exotic and native plants, but the biomass of exotic plants was positively affected by the presence of endogeic and epigeic earthworms as well as warming. Given the manifold interactions between earthworms and seeds, the exotic plant species used may have experienced co-evolutionary processes and may thus be able to benefit from the presence of earthworms (Forey et al. 2011). These differential responses of native and exotic plant species to warming and worming indicate that interactions of these global change agents are likely to alter the composition of herbaceous plant communities of North American forest ecosystems and affect their susceptibility to subsequent plant invasions. The invasion of anecic and epigeic earthworms will select for plant species able to germinate on bare soil, which most likely will detrimentally affect the regeneration of many native plant species of northern temperate forests. Warming will decrease soil water content, particularly in the presence of earthworms, and select for plant species more tolerant to drought during germination and early establishment phases.

Despite the bottleneck stage of the plant life cycle investigated, our results have to be treated with caution since we did not consider temporal (seasonal and daily) variations and long-term effects of warming and worming. Moreover, it should be noted that only a limited species pool of four native (of which only three germinated) and four exotic species was used. Although we had representatives of three major plant functional groups for both native and exotic plants, caution should be had in extrapolating these results to exotics and natives more broadly, as these results could be representative solely of this particular species set.

Conclusions

The present study shows that effects of exotic earthworms and warming interactively affected a bottleneck stage of plant regeneration through various mechanisms. These results, although not directly transferrable to natural conditions, suggest that the consequences of anthropogenic spread of exotic earthworms and warming would be more realistically understood if studied in concert. The invasion of epigeic and anecic species may select for plant species able to germinate on bare soil and tolerate dry soil conditions, with the latter being even more important in warmer environments. This could result in simplified plant communities with increased susceptibility to the invasion of exotic plants.

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Supplementary material (available as Appendix O19807 at <www.oikosoffice.lu.se/appendix>). Appendix 1.