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Subsurface earthworm casts can be important soil microsites that specifically influence the growth of grassland plants.

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1. ABSTRACT

Earthworms (Annelida: Oligochaeta) make up the majority of faunal biomass in temperate grasslands and are important ecosystem engineers because they bioturbate soil, process plant residues and organic matter, and produce nutrient enriched casts. By depositing several tons of nutrient-rich casts on the soil surface they create a spatial and temporal heterogeneity that can play a role in structuring plant communities. Earthworm casts are not only enriched in nutrients but can also contain spores and propagules of arbuscular-mycorrhizal fungi (AMF). However, while we begin to understand the role of surface casts we know virtually nothing about potential effects of subsurface casts.

In order to investigate the role of subsurface casts on grassland plants, we conducted a greenhouse experiment using large mesocosms (45 l volume) to test whether (i) soil microsites consisting of earthworm casts with or without AMF affect biomass production of eleven grassland plants comprising the three functional groups grasses, non-leguminous forbs and leguminous forbs, (ii) earthworms of the ecological groups endogeics (soil dwellers) or anecics (vertical burrowers) affect the influence of soil microsites.

Our results show that minute amounts of subsurface casts (0.89 g kg^{-1}) affect the biomass production of forbs and legumes but not of grasses. Nutrient-rich subsurface casts decreased root production of forbs and legumes. Earthworms reduced root biomass production of grasses only. We also found evidence that subsurface microsites can be initial locations from which root AMF colonization can start. Different ecological groups of earthworms did not differ in their effects on plant production or AMF distribution. These findings for the first time show that the often neglected subsurface earthworm casts could play a role in structuring plant communities by specifically affecting certain functional groups of plants.

Key-words: earthworms, grassland ecology, mesocosm experiment, plant-animal-fungi interaction, polymerase-chain-reaction (PCR), vesicular-arbuscular mycorrhizal fungi (AMF)

2. INTRODUCTION

In grasslands, earthworms are considered as ecosystem engineers because of their important effects on the structure and functioning of these ecosystems (Jones, Lawton and Shachak, 1994). During a year, earthworms can egest up to 250 tons ha^{-1} of nutrient-rich casts below and on the surface of soils (Bohlen 2002). On the soil surface casts are rather heterogeneously distributed showing associations with certain plant species and hence stimulating their growth (Zaller and Arnone 1999). Thus, earthworm casting activity may lead to spatially and temporally heterogeneous soil resources which can be specifically utilized by plant species (Jackson and Caldwell 1992; Bilbrough and Caldwell 1997, Farley and Fitter 1999), eventually affecting the structure of plant communities. However, while we have a limited understanding on interactions between earthworm surface casts and plant species (Zaller and Arnone 1999), we know virtually nothing regarding the functional significance of subsurface casts for plant species assemblages.

In temperate grasslands, three ecological groups of earthworms are distinguished: epigeic species which live in the top soil layer and build no permanent burrows, anecic species which build vertical burrows reaching from the soil surface down to the mineral soil layer and endogeic species which burrow within the upper mineral soil layer (Bouché, 1977). When studying anecic (*Lumbricus* spp.) and endogeic species (*Aporrectodea* spp.) it has been shown that, depending on temperature, up to 90% of earthworm casts are deposited at the soil surface (Whalen et al. 2004).

Earthworm casts not only contain much more nutrients than the surrounding soil (Edwards and Bohlen 1996), they can also contain considerably higher numbers of vesicular-arbuscular mycorrhizal fungi (AMF) spores and propagules than undigested field soil

(Reddell and Spain 1991; Gange 1993). Indeed, earthworms have been shown to feed on mycorrhizal structures (Hedlund and Augustsson 1995; Bonkowski et al. 2000), influencing AMF spore and hyphal distribution (Hedlund and Augustsson 1995) and mycorrhizal colonization of plant roots (Yu et al. 2005; Zarea et al. 2009). While mycorrhizal fungi enhance the phosphorus and nitrogen uptake of plants, the fungi are also dependent on plants as a host for their carbon nutrition (Smith and Read 2008). Therefore, plant species can differ in their degree of benefit they receive from different mycorrhizal symbionts (van der Heijden et al. 1998 b; Klironomos 2003; Zaller, Drapela and Frank, 2011).

In order to investigate the role of subsurface casts for grassland plant species, we conducted a full-factorial greenhouse experiment using 45 l mesocosms where artificial soil microsites consisting of earthworm casts with or without AMF were established. The experimental setup comprised eleven grassland plant species (grasses, non-leguminous forbs and leguminous forbs), two earthworm species comprising anecic and endogeic ecotypes and four AMF species of the taxon *Glomus*. We hypothesized that (i) different functional groups of grassland plants differ in their ability to utilize nutrient-rich subsurface earthworm casts, (ii) different earthworm ecological groups due to their different feeding and burrowing habits have different effects on AMF distribution and plant growth, and (iii) AM fungi present in soil microsites will be more efficiently distributed among plants by the activity of earthworms.

3. MATERIAL AND METHODS

3.1 Experimental setup and treatments

We conducted the experiment using plastic pots (diameter 45 cm, volume 45 l; further called mesocosms) in a greenhouse of the University of Natural Resources and Life Sciences Vienna from August 2008 until March 2009. Mesocosms were filled with 40 l of a 1:2 vol./vol. mixture of field soil (Haplic Chernozem, silty loam) and quartz sand (mean grain 1.4 - 2.2 mm). Characteristics of this mixture was: pH = 7.6, C_{org} = 2.2 ± 0.2 %, N_{tot} = 0.102 ± 0.003%, K = 119.3 ± 0.9 mg kg^{-1} and P = 62.33 ± 0.33 mg kg^{-1} (Zaller et al. 2011a). Field soil was obtained from an arable field at the University's Research Farm Groß-Enzersdorf, sieved (mesh size: 1 cm), mixed with fire-sterilized quartz sand, and steam-sterilized (110°C for 3 hours). This soil-quartz sand mixture has been successfully used in previous experiments including the same plant, earthworm and AMF taxa (Putz *et al.*, 2011; Heiner *et al.*, 2011; Zaller et al. 2011a).

The three-factorial experimental design consisted of the factor earthworms (four levels: addition of only endogeic worms – Apo, addition of only anecic worms – Lum, addition of a mix of endogeic and anecic worms – ApoLum, no earthworm addition – - EW), subsurface microsite (two levels: microsite created of earthworm casts – +cast; microsite created of soil only without casts – -cast) and AMF inoculation (two levels: inoculation of microsites with a mix of four active *Glomus* taxa – +AMF; inoculation of subsurface microsites with a mix of four sterilized *Glomus* taxa – -AMF; Fig. 1). More details on the taxa used can be found below. Each treatment was replicated six times making up a total of 96 mesocosms (4 earthworm x 2 subsurface microsites x 2 AMF treatments x 6 replicates).

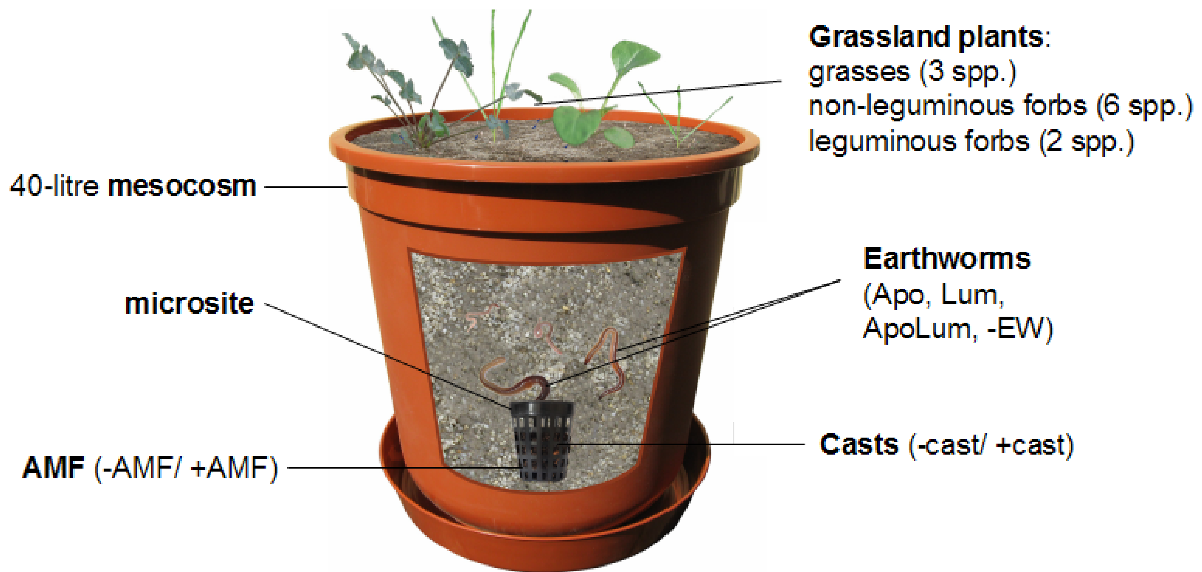


Figure 1. Schematic overview of the experimental setup to test the effects of earthworms (Apo... endogeic *A. caliginosa*, Lum...anecic *L. terrestris*, ApoLum...mixture of *A. caliginosa* and *L. terrestirs*, -EW...no earthworms added) on the grassland plant utilization of soil microsites with/without earthworm castings and/or arbuscular mycorrhizal fungi inoculum (-AMF/+AMF); modified after Heiner et al. 2008.

In order to prevent earthworms from escaping, the drainage holes of the pots were covered with water-permeable fleece material, additionally a 20 cm high barrier of transparent plastic coated with soft soap on the upper 2 cm was attached to the upper rim of the pots. Fleece and barriers were also installed on pots containing no earthworms to ensure similar microclimatic conditions between different treatments.

3.2 Earthworm inoculation

We used the anecic *Lumbricus terrestris* L. and the endogeic *Aporrectodea caliginosa* (Savigny 1826) species; both species are common in temperate grasslands throughout Europe. Treatment Apo received 10 individuals (in total 5.7 ± 0.14 g fresh weight) of adult/subadult A. *caliginosa*, treatment Lum received 2 individuals (in total 8.3 ± 0.15 g fwt) of adult *L. terrestris*, treatment ApoLum received 5 individuals of *A. caliginosa* and 1 individual of *L.*

terrestris (in total 3 ± 0.09 g fwt plus 4.4 ± 0.21 g fwt for Apo + Lum, respectively) (Table 1). *A. caliginosa* was collected by hand-digging from an agricultural field (Landwirtschaftliche Bundesversuchswirtschaften Königshof, Wilfleinsdorf, Austria), *L. terrestris* was obtained from a commercial supplier (Denuwurm, Stuttgart, Germany). All earthworms were kept in a climate chamber (15°C) in sterilized soil for two weeks before they were inserted to the mesocosms to allow the egestion of potential AMF from the field site, carefully rinsed with tap water before they were introduced to the mesocosms.

3.3 Planting

Each mesocosm was planted with 11 low-fertile grassland species, representing the three major groups grasses (*Arrhenatherum elatius* L., *Bromus erectus* Huds., *Dactylis glomerata* L.), non-leguminous forbs (*Hieracium pilosella* L., *Knautia arvensis* (L.) Coult., *Leucanthemum vulgare* LAM., *Plantago lanceolata* L., *Prunella vulgaris* L., *Salvia pratensis* L.) and leguminous forbs (*Lotus corniculatus* L., *Trifolium pratense* L.). Seeds were obtained from a commercial supplier (Rieger-Hofmann GmbH, Blaufelden, Germany) and germinated on a wet filter paper before transplanted into the mesocosms in a regular pattern along two concentric circles. The outer circle had a diameter of about 32 cm and contained two individuals of each plant species, with the exception of *A. elatius* which was planted with four individuals on two spots with two individuals each. The inner circle had a diameter of about 16 cm and contained one individual of each species with the exception of *A. elatius* which was planted two times. In total 24 plants were planted in each mesocosm. Dead plants were consistently replaced by new seedlings during the first weeks of the experiment. The mesocosms were watered when needed using a constant amount of tap water.

3.4 Installation of soil microsites

One week after planting, we inserted one microsite at 25 cm soil depth in the middle of every mesocosm using a 5-cm diameter corer. The microsite consisted of a plastic grid pot (diameter 5 cm, height 7 cm; mesh size about 1 cm) commonly used for aquarium plants. Using these grid pots helped to create compact microsites, while enabling earthworms and roots to enter and facilitating the location of the microsite during the final harvest of the mesocosms. According to the specific treatments these microsites contained either 50 g of casts produced by earthworms of the respective treatment (i.e. for Apo treatment, casts were produced by *A. caliginosa*; for Lum treatment by *L. terrestris;* for ApoLum treatments 25 g of casts produced by either *A. caliginosa* or *L. terrestris* were mixed). Therefore, 10 individuals of *A. caliginosa* or 2 individuals of *L. terrestris* or the combination of both were separately held in plastic boxes containing 500 g field soil and regularly fed with ground oat meal in order to produce casts; 5 replicates of this setting were prepared. After two weeks all soil in the boxes was readily ingested and excreted again so that all material in the boxes consisted of casts. This material was then filled into the grid pots and inserted into the microcosms.

In –cast treatments these microsites contained sterilized field soil only. In +AMF treatments, microsites additionally contained 25 g of AMF inoculum; the same amount of sterilized inoculum was added to the -AMF treatments. The inoculum consisted of a mixture of clay granules, infected root pieces and AM fungi spores of *Glomus claroideum*, *G. intraradices*, *G. mossae* and *G. geosporum* (Symbio-m, Lanskroun, Czech Republic). Each microcosm also received 10 ml of a microbial wash prepared by wet-sieving 1000 g inoculum and 3000 g of field soil through a series of sieves (finest sieve was 10 µm) into a final volume of 960 mL to correct for possible differences in microbial communities between field soil and sterilized soil mixture (Koide and Li 1989).

We took three subsamples of each microsite treatment and analysed their inorganic ammonium-N and nitrate-N concentration in 0.5 M K_2SO_4 extracts (1:5 weight:volume) using

the modified indophenol blue technique (Sims, Ellsworth et al. 1995), with a Bio-Rad Microplate Reader 550 (Table 1).

Table 1 Ammonium-N and nitrate-N concentrations in subsurface microsites containing casts from different earthworm species or the experimental soil mixture only. ANOVAs revealed no significant differences in nutrient contents between earthworm treatments or AMF, but significant differences between soil-only microsites and microsites containing casts. Means ± SE, n = 3.

Microsite composition	**NH_4^+-N**		**NO_3^--N**		**NH_4^++NO_3^--N**	
	-AMF	**+AMF**	**-AMF**	**+AMF**	**-AMF**	**+AMF**
Apo casts	17.38 ± 9.69	15.57 ± 5.87	23.02 ±10.32	33.24 ± 9.12	40.4 ± 20.00	48.8 ± 14.90
ApoLum casts	12.66 ± 6.60	12.95 ± 4.62	15.90 ± 3.86	20.61 ± 4.21	28.56 ± 9.16	33.56 ± 8.82
Lum casts	10.97 ± 3.60	13.10 ± 4.25	18.30 ± 5.69	22.1 ± 5.62	29.27 ± 6.87	35.2 ± 6.88
Only soil (control)	4.23 ± 2.07	6.56 ± 1.22	9.22 ± 1.46	10 ± 2.18	13.45 ± 0.75	16.56 ± 1.13

3.5 Monitoring plant growth and earthworm activity

Because the rapidly growing grasses *A. elatius* and *D. glomerata* would overgrow all other plants in the mesocosms, we cut them at 2 cm above soil surface 13 weeks after starting the experiment.

Earthworm surface casts were collected once a month, dried at 40°C for 24 hours and weighed. Occasionally, we found dead earthworms lying on the soil surface; those were replaced immediately with new specimens after quarantine in sterile soil. We also regularly sampled plant roots from each mesocosm using a 1-cm soil corer to check for the presence of AMF colonization under the microscope after staining with vinegar and ink (Vierheilig et al., 1998).

3.6 Harvest

Mesocosms were harvested destructively six months after the start of the experiment by flipping over the pots and carefully taking out the individual plants. Maximum shoot lengths were measured before aboveground parts were dried at 40°C to determine their dry mass. Roots were cut off and stored at 4°C until they were washed free of soil using tap water, dried at 40°C and weighed. For AMF DNA analysis (see below), we randomly selected roots from *T. pratense*, *A. elatius*, *S. pratensis*, *B. erectus*, *P. lanceolata* and *L. vulgare* from three replicates of each treatment. Plant roots which grew into the microsites were sampled from the same three replicates of each treatments, to estimate AMF root colonization. Roots growing into microsites were sorted out, dried at 50°C for 24 hours and weighed. Root parts that could not be assigned to a species were collected and considered as bulk roots in order to determine total mesocosm root production. At harvest, all live and dead earthworms were counted and weighed.

3.7 Analysis of AMF-DNA in plant roots and aboveground earthworm casts

Root and cast samples were oven-dried (60 °C) and milled to a fine powder in a beat beater (FastPrep120, Bio101) with Lysing Matrix A (MP Biomedicals GmbH). DNA was extracted and purified from the powder by LGC Genomics (Germany). A 1:20 dilution of the DNA was used as a template. For sensitive detection of the four *Glomus* species from the inoculum in root and cast samples a nested PCR was performed. The first amplification was carried out with two *Glomu*s-specific forward primers (GlGrA and GlGrB, Schüßler et al. 2001) and a universal reverse primer (SSU-1536-3‘ (Borneman and Hartin 2000). The second amplification step made use of the primer pair AM1/NS31 (Helgason et al. 1998; Simon et al. 1992). The PCR conditions were as follows: 10 µl 2 × GoTaq Green Master Mix (Promega), 10 µg BSA, 20 pmol forward primer, 20 pmol reverse primer, 0.5 µl template DNA and 20 µl ultrapure water added. Cycling parameters for the first PCR were: initial denaturation at 95 °C

for 2‘30“ followed by 30 cycles of denaturation at 94°C for 30“, annealing at 57°C for 30“ and extension at 72°C for 1“, and a final extension at 72°C for 5‘. For the nested PCR the following cycling parameters were changed: the annealing temperature was raised to 60°C and the extension time was shortened to 30“. PCR-products were separated on an agarose gel and visualized under UV. Quality control of sample DNA was performed with the fungal specific primer pair ITS1F/ITS4 (Gardes and Bruns 1993; White et al. 1990) and cycling parameters as above except that anneling temperature was set to 54°C, extension time to 45“ and cycle number to 35. From all root and cast samples fungal DNA could be amplified.

3.8 Statistical analysis

The biomass data (root and shoot dry weight) of the plant individuals were averaged per species and mesocosm in order to prevent pseudoreplication. For generating the biomass data per plant group (grasses, forbs, legumes), the values of the respective species were summed up. Data of plant biomass, earthworm fresh mass and earthworm activity are given as means per mesocosm ± standard error of the mean (SE).

Effects of the factors “Casts” (+/- earthworm casts in the microsites), “AMF” (+/- AMF inoculate in the microsites) and “Earthworms” (+/- three different earthworm population treatments in the mesocosms) on plant biomass and earthworm performance (fresh weight and cast activity) were analysed by three-factorial ANOVAs. In case of the factor “Earthworms” Tukey *post-hoc* tests were conducted. When analysing the earthworm data, only the mesocosms containing earthworms were included. If significant interactions between two factors were detected, 2-factorial ANOVAs were calculated for each level of one of the interacting factors to be able to detect differences between factor levels within the respective factor combinations.

Relationships between plant biomass data and earthworm activity were described and tested by Pearson correlation coefficients.

Effects of the factors “Casts” and “Earthworms” on the measured mycorrhization rates inside

and outside the microsites were analysed by Chi-squared-tests.

Normal distribution was tested by the Kolmogorov–Smirnov-Test, homogeneity of variances by the Levene-Test. If necessary, data were transformed by natural logarithm to improve normality and homogenize the variances (Köhler et al. 2002).

All statistical analyses were performed with SPSS 11.5.1 (SPSS Inc., Chicago, IL, USA).

4. RESULTS

Root and shoot biomass of both, forbs and legumes, was significantly lower in mesocosms with soil microsites containing casts (+cast) than in mesocosms containing soil microsites without (-cast; Figure 2b,c; Table 2). Contrary to that, neither root biomass nor shoot biomass of grasses was affected significantly by cast treatments (Table 2, Figure 2a). Soil microsites were utilized by plant roots, however there was no difference among the treatments in root biomass present within the soil microsites (data not shown). Plant biomass production was, except for grass roots, not affected by earthworms (Table 2, Figure 2a). AMF in soil microsites significantly decreased total grass shoot biomass due to affecting *A. elatius*, however did not affect biomass production of other plant species (Table 2, Figure 2a).

At the destructive harvest of the experiment we found 58% of the initial biomass in Apo-treatments and 95% in Lum-treatments. Within ApoLum-treatments, 31% of earthworm biomass was recovered. Above ground casting activity was consistently high throughout the entire experiment and highest in treatments which contained *L. terrestris*. Casting activity of *A. caliginosa* clearly increased within the last two months of the mesocosm experiment. At the same time cast production of *L. terrestris* decreased (data not shown). Neither number nor mass of surface cast production was affected by earthworm treatments or the presence of AMF in subsurface microsites (Table 3, Figure 3b and c). However, both number and mass of surface casts were significantly lower when casts were present in subsurface microsites.

There was a significant positive correlation between forb root mass and above ground cast production (dry weight) in Lum and ApoLum- treatments (Figure 4).

Table 2. ANOVA table (F-values) of the effects of earthworm treatments (EW), mycorrhiza inoculation (AMF) and earthworm cast amendment (CAST) on plant biomass parameters (DM of shoot and roots) of grasses (*Arrhenaterum elatius, Dactylus glomerata, Bromus erectus*), forbs (*Leucanthemum ircutianum, Knautia arvensis, Hieracium pilosella, Prunella vulgaris, Salvia pratensis, Plantago lanceolata*) and legumes (*Trifolium pratense, Lotus corniculatus*): *P < 0.05, **P < 0.01

	Source of variation						
Dependent variable	EW	AMF	CAST	EW x AMF	EW x CAST	AMF x CAST	EW x AMF x CAST
Grasses							
Total grass shoots	0.305	**5.537***	0.323	0.286	1.235	0.040	0.552
Total grass roots	**3.396***	0.547	0.000	0.420	0.117	1.183	1.336
A. elatius shoots	1.297	**6.592***	0.832	0.354	0.795	0.697	0.078
A. elatius roots	1.826	1.386	0.008	0.523	0.252	2.691	0.439
D. glomerata shoots	1.644	0.549	1.869	1.068	1.174	0.539	1.528
D. glomerata roots	1.300	0.218	0.402	0.458	0.816	0.042	2.186
B. erectus shoots	0.771	0.010	2.897	0.801	0.151	0.731	0.378
B. erectus roots	1.621	0.742	1.312	0.180	0.256	0.013	0.490
Forbs							
Total forb shoots	0.069	0.488	**9.835****	2.084	0.878	0.011	0.733
Total forb roots	0.222	1.183	**12.842****	**3.204***	1.130	0.318	1.433
L. ircutianum shoots	0.491	0.314	2.504	1.608	0.209	1.290	2.373
L. ircutianum roots	0.299	0.351	3.768	2.398	0.252	2.203	2.652
K. arvensis shoots	2.309	0.062	**7.814****	1.140	0.992	1.509	1.453
K. arvensis roots	0.884	1.621	**6.372***	1.715	2.014	0.213	**3.336***
H. pilosella shoot	2.534	0.145	0.811	0.411	1.089	0.111	0.280
H. pilosella roots	2.587	0.026	0.139	1.353	0.938	0.533	0.148
P. vulgaris shoots	0.913	0.458	2.365	2.133	**5.061****	2.623	1.490
P. vulgaris roots	1.466	3.866	1.330	0.693	**4.007***	0.496	0.067
S. pratensis shoots	2.531	0.554	**5.466***	0.400	0.802	0.246	1.121
S. pratensis roots	1.107	0.073	**6.087***	0.739	0.544	1.297	0.467
P. lanceolata shoots	0.230	0.000	**6.856***	2.077	1.224	1.761	0.400
P. lanceolata roots	0.228	0.014	**5.968***	2.461	1.041	1.861	0.634
Legumes							
Total legume shoots	0.922	0.000	**6.226***	0.400	0.692	0.072	0.526
Total legume roots	1.647	0.000	**6.385***	2.368	1.559	1.195	0.940
T. pratense shoots	0.526	0.008	**5.215***	0.224	0.972	0.001	0.852
T. pratense roots	0.921	0.049	**4.207***	0.645	1.157	2.057	1.397
L. corniculatus shoots	1.122	0.091	1.774	1.012	**4.008***	1.423	1.957
L. corniculatus roots	0.360	1.618	**5.147***	**4.293****	0.327	0.049	0.880

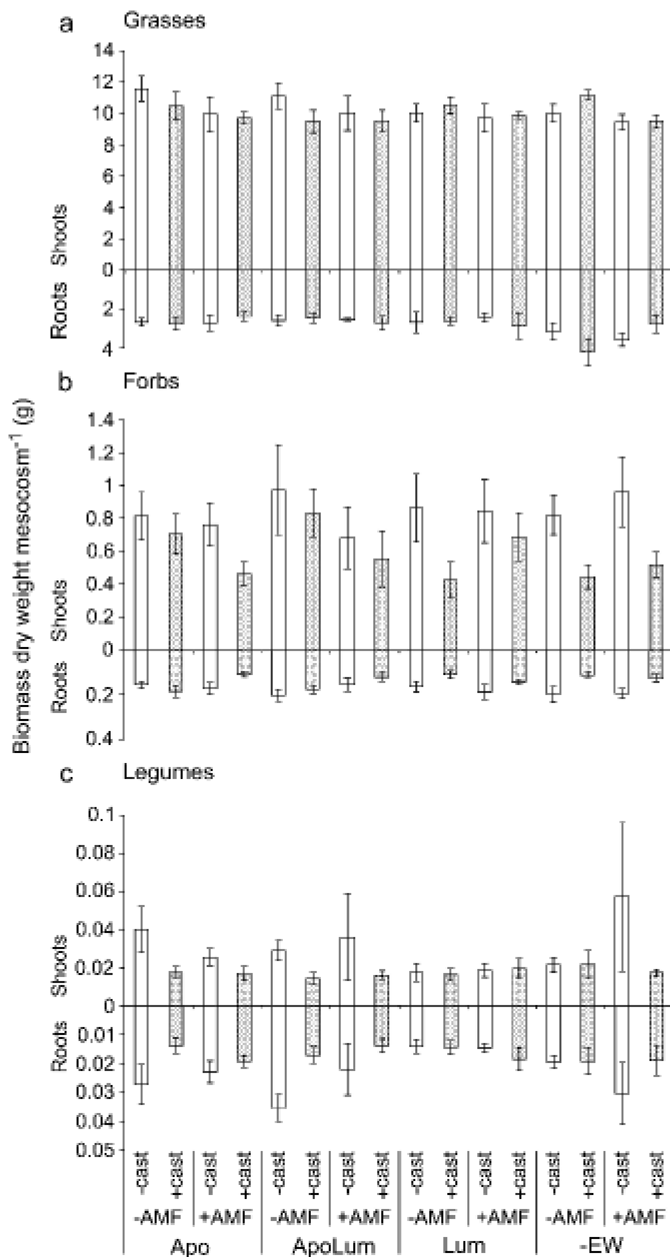
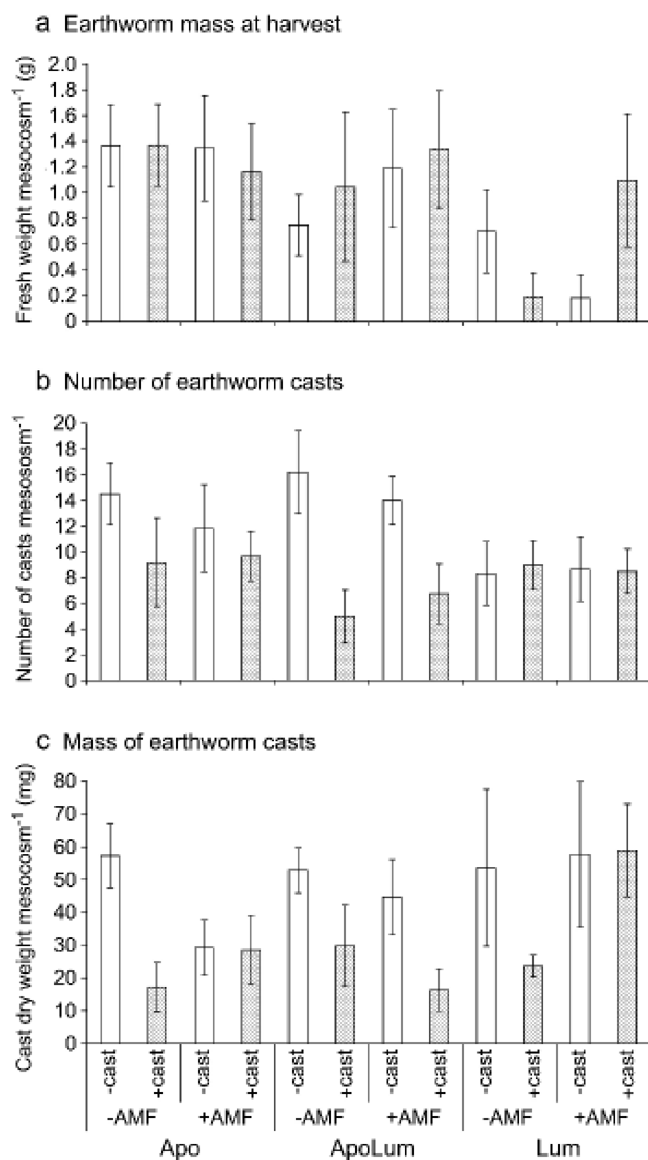


Figure 2. Shoot and root dry mass of plant functional groups in response to earthworms (Apo...*A. caliginosa* only, Lum...*L. terrestris* only, mix between the two spp., -EW...no earthworms) and soil microsites with arbuscular mycorrhizal fungi (AMF, -AMF...no AMF inoculation, +AMF...with AMF inoculation) and/or earthworm casts (-cast...no casts, +cast...with cast amendment). Means ± SE, n = 6.

Table 3 ANOVA table (F-values) of the effects of earthworm treatments (EW), soil microsites amended with mycorrhiza inoculation (AMF) and/or earthworm casts amendment (CAST) on earthworm biomass and cumulative earthworm cast activity. Only treatments containing earthworms were considered in the analysis. *P < 0.05, **P < 0.01

	Source of Variation						
Dependent variable	**EW**	**AMF**	**CAST**	**EW x AMF**	**EW x CAST**	**AMF x CAST**	**EW x AMF x CAST**
Earthworm mass, harvest	**4.235***	0.452	0.259	0.402	0.207	0.674	1.436
Earthworm casting activity							
Number of surface casts	1.272	0.106	**8.664****	0.050	**3.578***	0.524	0.270
Mass of surface casts	1.512	0.000	**6.552***	1.594	0.170	1.900	0.728



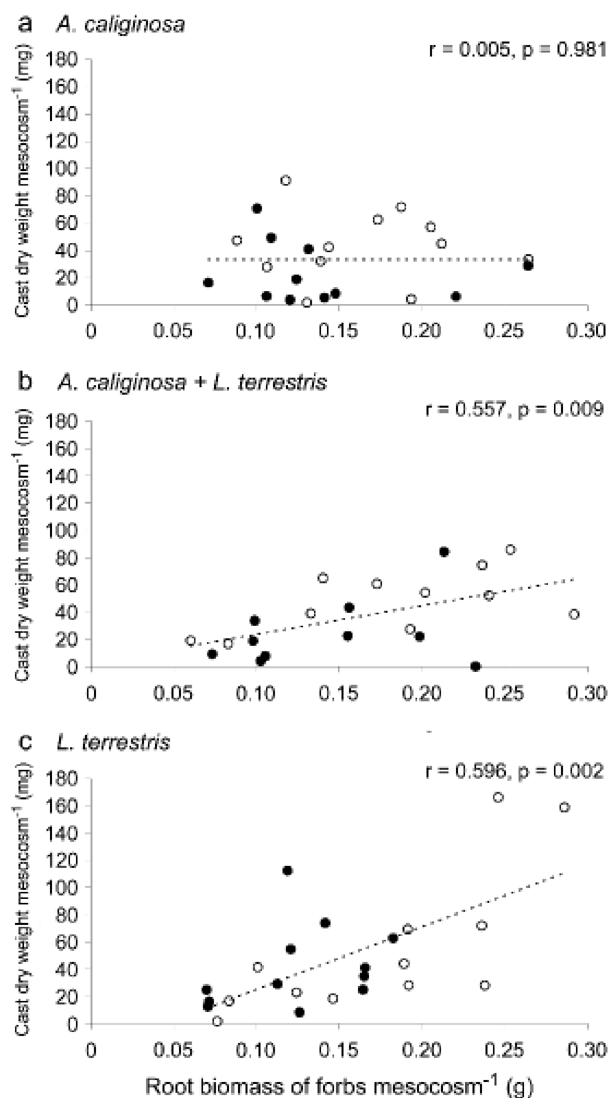


Figure 3. Earthworm fresh mass at harvest and above ground cast production in response to earthworm treatments (Apo… *A. caliginosa*, Lum…*L. terrestris*, ApoLum… *A. caliginosa* and *L. terrestris*) and soil microsites containing inoculum of arbuscular mycorrhizal fungi (-AMF… no AMF, +AMF… AMF inoculation) and earthworm casts (-cast….no casts, +cast…with casts). Means ± SE, n=6.

Figure 4. Pearson correlation between cumulative surface cast production of different earthworm treatments and forbs root biomass at harvest, n=6.

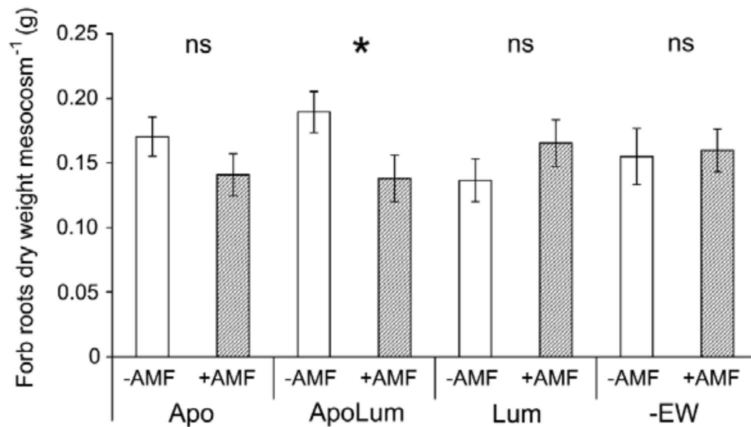


Figure 5. Forbs root dry weight in response to earthworm treatments (Apo… *A. caliginosa*, Lum…*L. terrestris*, ApoLum… *A. caliginosa* and *L. terrestris,* -EW…. no earthworms) and presence of arbuscular mycorrhizal fungi in soil microsites (-AMF… no AMF, +AMF… AMF inoculation) ± SE, n=6. *denotes significant difference, ns...difference not significantly different.

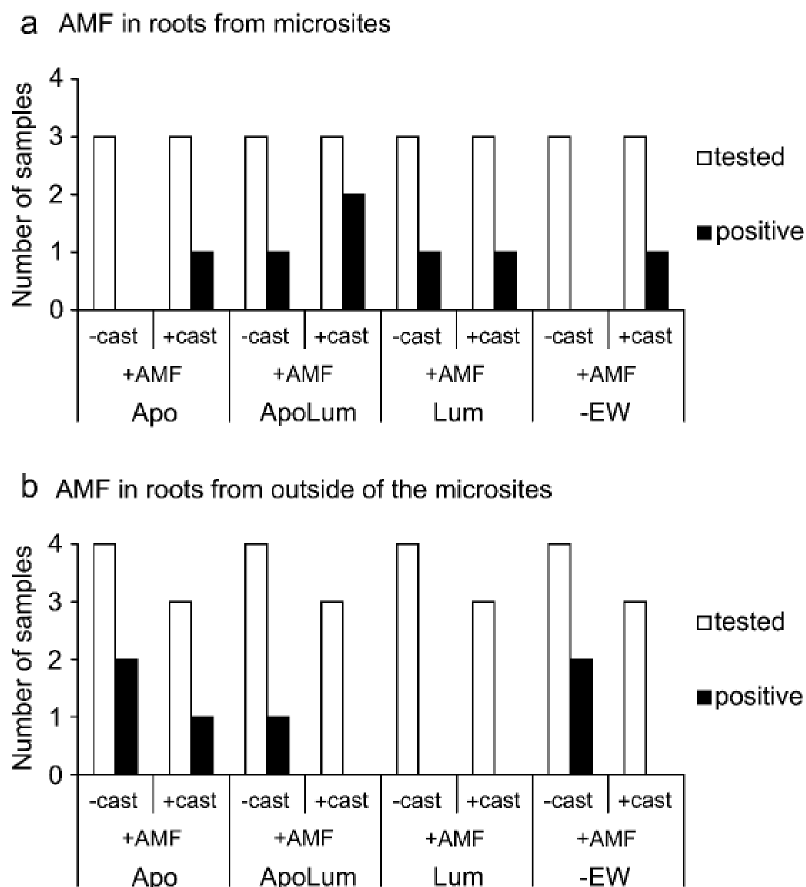


Figure 6. Total AMF- detection using PCR of selected plant species (*T. pratense*, *A. elatius*, *S. pratensis*, *B. erectus*, *P. lanceolata* and *L. vulgare*) in response to earthworm treatments (Apo...*A. caliginosa*, Lum...*L. terrestris*, ApoLum...*A. caliginosa* and *L. terrestris*) and soil microsites containing inoculum of arbuscular mycorrhizal fungi (-AMF...no AMF, +AMF...AMF inoculation) and earthworm casts (-cast...no casts, + casts...with casts) within microsites (a) and mesocosms (b). Missing columns mean that no AMF was detected.

There was a significant interaction between earthworm and AMF treatments regarding their effects on forb roots (Table 2). Within ApoLum treatments mycorrhizal fungi in soil microsites decreased forb root biomass (Figure 5). In contrast, AM fungi in soil microsites did not affect forb root biomass in other treatments (Figure 5). Aboveground biomass production of grasses, particularly of *A. elatius*, was significantly lower when subsurface microsites contained AMF (Table 2). No AMF could be detected in roots by the staining technique, which indicates very low colonization rates.

The rate of AMF detection in roots was low: out of 192 root subsamples taken and tested on mycorrhization using PCR, only 16 samples (8.3%) were tested positive (Figure 6). AMF detection rates were not affected by earthworms or cast presence in microsites (roots im microsites: Cast: $p= 0.317$, EW: $p= 0.766$; roots outside of microsites: Cast: $p= 0.196$, EW: $p=0.430$). However, there was a pattern towards more frequent AMF detection in treatments where no casts were present in soil microsites. This trend was not recognizable within subsamples taken from microsites (Figure 6).

5. DISCUSSION

This study for the first time shows that subsurface earthworm casts are important soil microsites that can specifically influence forbs and legumes but not grasses in model grasslands. This is especially interesting as these effects were caused by only 50 g of earthworm casts located in 25 cm depth within 56,000 g of soil in the experimental mesocosms. Effects on plants were little influenced by different ecological groups of earthworms. Results also suggest that AMF within these subsurface microsites could colonize plant roots.

5.1 Subsurface casts and plant production

Shoot and root biomass of forbs and legumes was found to be lower in +cast treatments while grass biomass production remained unaffected. The lack of response to casts of grasses is somewhat surprising as earlier studies showed a positive correlation between surface casts and grasses (Tomati et al. 1988, Zaller and Arnone 1999). Despite the unresponsiveness to additional nutrients provided by subsurface casts grasses were the plant group with the highest biomass production above and below ground in the experiment. Thus, we conclude that grasses, due to their large and fast growing root systems, exploited the available soil volume faster than forbs or legumes being less dependent on the nutrient-rich microsites (Farley and Fitter 1999; Zaller 2007). During the course of the experiment grass roots in both cast treatments grew through the obtainable soil while forbs and legumes within the +cast treatments suffered from the early competitive pressure and stayed smaller than in the –cast treatments. Competition during critical developmental stages of plants or plant groups may prevent highest possible biomass production (Hooper 1998).

The presence of roots within microsites showed that these spots were indeed utilized by plants. The biomass of roots which grew into the microsites did not differ between different soil microsites despite the fact that nutrient concentrations in microsites with casts were considerably higher than in microsites containing soil only. It might well be that more fine roots grew into cast microsites but could not be detected by measuring root biomass only. We and others (Springett and Syers 1979, Spiers et al. 1989, Zaller and Arnone 1999b) have observed root proliferation into casts, indicating that plants can exploit these nutrients. Differences in the abilities of species to exploit these nutrient patches (Jackson and Caldwell 1992, Bilbrough and Caldwell 1995) may alter the competitive balance among plant species in plant communities. The finding that the presence of subsurface casts significantly decreased earthworm surface cast production both in cast numbers and mass can be explained by several direct and indirect effects. First, aside from plant roots, microorganisms associated with subsurface casts might be an additional food source

for earthworms (Curry and Schmidt 2007). Particularly the endogeic earthworm *A. caliginosa* might prefer to utilize a subterraneous food source and therefore deposits casts less frequently on the soil surface. Second, subsurface casts decreased root mass production, therefore earthworms feeding on roots spent more time in the soil without depositing casts on the surface. Indeed, a significant negative correlation between forb root mass and surface cast mass indicate that roots stimulated earthworm activity. Similar correlations have already been reported elsewhere (Shipitalo et al. 1988; Zaller and Arnone 1997). Vice versa, root herbivory by earthworms could also stimulate root production (Gange and Brown 2002). We found some indication for this as in the three earthworm treatments (Apo, Lum and ApoLum) at least the root biomass of the grasses was significantly lower than in the treatment without earthworms. The reason for that might be a negative effect of earthworms on plant root biomass by their feeding behaviour, however this remains to be investigated in more detail.

We observed a marked decline of earthworm biomass during the course of the experiment in all earthworm treatments. The biomass of *L. terrestris* was found to be significant lower at the time of harvest than the biomass of *A. caliginosa*, despite an inverse weight ratio at the start of the experiment. Such declines are frequently observed in earthworm laboratory studies, especially when experiments lasted several months (Wurst, Dugassa-Gobena et al. 2004, Zaller et al. 2011b). Based on the fact that earthworm casting activity changed little until the end of the experiment we assume that (i) a decline of earthworm individuals and their loss of weight occurred within the last week before harvest when we discontinued watering to facilitate harvesting, (ii) furthermore we admit that during destructive harvest several worms might have been overlooked in the relatively large mesocosms.

5.2 Effect of AMF in subsurface microsites

Overall, the rate of AMF detection using PCR was low throughout our experiment. We attribute this mainly to the fact that only a minute amount of AMF inoculum was added on a single spot to the soil mass (0.89 g kg^{-1}) in the comparatively large (45 liters) mesocosms. Knowing that the presence of soil microorganisms such as *Azotobacter* and *Pseudomonas* can produce growth substances that can increase the mycorrhizal colonization of plants (Azcon et al. 1978; Bagyaraj and Menge 1978; Brown and Carr 1979), we amended a microbial wash to all mesocosms. Nevertheless, given this small amount of inoculum within the mesocosms it is remarkable that AM fungi significantly affected (reduced) the shoot biomass of the grasses. The relationship between mycorrhizal fungi and plants was described as a mutualism-parasitism continuum related to the cost or benefit which is received by the plants (Hayman 1982; Fitter 1991). We conclude that the AMF-plant symbiosis at time of harvest tended to have parasitic traits resulting in a decreased plant biomass but could have developed to a mutualistic relationship over the time.

Subsurface casts tended to increase AMF detection rate in roots within microsites, a finding that confirms studies stating that cast material provides a favorable environment for AMF containing up to 10 times more spores and infective propagules than nearby field soil (Gange 1993). Subsurface casts however reduced AMF in roots outside of the microsites. This is probably due to the increased availability of nutrients as the outcomes of several studies indicated that AMF root colonization was reduced when N and P were available in sufficient concentrations for the plants (Abbott et al. 1984; Liu et al. 2000, Johnson et al. 2003). Another explanation for the increased distribution of mycorrhizal fungi within the -cast treatments could be that earthworms acted as vectors for AMF spores and the associated increased earthworm activity enhanced the distribution of mycorrhizal fungi. However, AMF infected roots outside the microsites were also found in mesocosms without earthworms restricted to the –cast treatment. Consequently, the enhanced earthworm activity within the –cast treatments can only

partly explain the positive effect on the measured mycorrhization rate. Root biomass of forbs was shown to be higher within the –cast treatments. The diminished distance of longer forb roots to the inoculate within the microsite might be another reason for the higher mycorrhization rate in these treatments. In this case, the competition between plant groups might have an impact on the progress and interaction of the functional groups within an successional system.

The only significant interaction between earthworms and AMF was seen on forb roots when anecic and endogeic earthworms were active. Whereas forb root biomass was reduced by the presence of AM fungi and earthworms present, this effect was not observed within treatments without earthworms (-EW) or in treatments which just contained anecic *L. terrestris* (Lum; Figure 4). Others also observed a reduction of plant biomass in systems which contained both earthworms and AMF because earthworms reduced the positive effect of AMF on root biomass within the symbiosis (Milleret et al. 2009, Zaller, Heigl et al. 2011, Zaller, Saccani et al. 2011). Interactions between earthworms and AMF on plant production might depend on the behaviour of the respective earthworm species. It is possible that mycorrhizal plant roots have a similar effect on the earthworm feeding behaviour (Bonkowski 2000). In our case we suggest that particularly *A. caliginosa* reduced root biomass of forbs within AMF treatments as a result of their preference for mycorrhizal forb roots. This suggestion is based on the higher mycorhization rate in treatments which contained *A. caliginosa* and the fact that mycorrhizal DNA was only found in an Apo- cast. Due to their endogeic mode of life *A. caliginosa* might have more effects on mycorrhizal fungi than *L. terrestris*. However, plant roots within –EW treatments were tested positive on AMF-DNA positive as well.

5.3 Conclusions

Taken collectively, our results demonstrate that (i) patchily distributed subsurface earthworm casts can potentially affect early successional grassland plant communities by altering the competition between plant groups, (ii) different earthworm functional groups seemed to have

similar (few) effects on plant biomass production showing little interaction with subsurface casts concerning plant biomass production; (iii) there is some indication that AM fungi located in subsurface casts are utilized by different plant species, however earthworms only seem to play a minor role in distributing AMF among plants. All in all the present results corroborate other studies on interactions between plants and earthworm casts (Zaller and Arnone 1999). The next challenge will be to understand the roles of both surface and subsurface earthworm casts in the natural environment. Methodological approaches that could help tracking these interactions have recently been suggested (Heiner, Drapela et al. 2011, Putz, Drapela et al. 2011).

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8. APPENDIX

8.1 Tables and Figures

FIGURE 1: **Schematic overview of the experimental setup**

TABLE 1: **Ammonium-N and nitrate-N concentrations in subsurface microsites**

TABLE 2: **ANOVA table (F-values) of the effects of earthworm treatments, mycorrhiza inoculation and earthworm cast amendment on plant biomass parameters (DM of shoot and roots) of grasses, forbs and legumes.**

FIGURE 2: **Shoot and root dry mass of plant functional groups in response to earthworms and soil microsites with or without arbuscular mycorrhizal fungi and/or earthworm casts.**

TABLE 3: **ANOVA table (F-values) of the effects of earthworm treatments, mycorrhiza inoculation and earthworm cast amendment in soil microsites on earthworm biomass at harvest and cumulative earthworm cast activity.**

FIGURE 3: **Earthworm fresh mass at harvest and above ground cast production in response to earthworm treatments and soil microsites containing inoculum of arbuscular mycorrhizal fungi and/or earthworm casts.**

FIGURE 4: **Pearson correlation between cumulative surface cast production of different earthworm treatments and forb root biomass.**

FIGURE 5: **Forb root dry weight in response to earthworm treatments and presence of arbuscular mycorrhizal fungi in soil microsites.**

FIGURE 6: **Total AMF- detection using PCR of selected plant species in response to earthworm treatments and soil microsites containing inoculum of arbuscular mycorrhizal fungi and earthworm casts within microsites (a) and mesocosms (b).**

8.2 Zusammenfassung

Regenwürmer (Annelida: Oligochaeta) machen den Großteil der tierischen Biomasse in Graslandökosystemen der gemäßigten Zone aus. Sie gelten als wichtige „Ökosystem-Ingenieure“ da sie den Boden durchmischen, totes Pflanzenmaterial verarbeiten und eine mit pflanzenverfügbaren Nährstoffen angereicherte Losung produzieren. Durch die Ablage von mehreren Tonnen nährstoffreicher Losung an der Bodenoberfläche schaffen Regenwürmer eine räumliche und zeitliche Heterogenität die die Struktur von Pflanzengemeinschaften beeinflussen kann. Die Regenwurmlosung ist allerdings nicht nur reich an Nährstoffen, sondern kann auch Sporen und Diasporen von arbuskulären Mykorrhizapilzen (AMF) enthalten. Während wir die Rolle von Regenwurmlosung an der Bodenoberfläche bereits zu verstehen beginnen, ist uns noch kaum etwas über mögliche Effekte unterirdisch abgelegter Regenwurmlosung bekannt.

Um die Rolle von unterirdisch abgelegter Regenwurmlosung in Graslandsystemen zu untersuchen, führten wir einen Glashaus-Versuch durch, in welchem wir mittels großer Mesokosmen (45 L Volumen) testeten ob (i) Boden-Mikrostandorte, bestehend aus Regenwurmlosung mit oder ohne AMF bestehen, die Biomasseproduktion von 11 Grasland-Pflanzenarten (drei funktionellen Gruppen: Gräser, Kräuter und Leguminosen), beeinflussen, (ii) endogäische oder anözische Regenwürmer den Einfluss von Microstandorten in Böden beeinflussen.

Die Ergebnisse zeigen, dass bereits kleine Mengen an Regenwurmlosung im Boden (0.89 g kg^{-1}) die Biomasseproduktion von Kräutern und Leguminosen, nicht aber jene von Gräsern beeinflussen können. Nährstoffreiche Regenwurmlosung die im Boden abgelegt wurde, verminderte die Biomasseproduktion der Leguminosen und Kräuter. Regenwürmer hingegen verminderten die Wurzelbiomasse der Gräser. Es wurden außerdem Hinweise daraufhin gefunden, dass unterirdisch deponierte Regenwurmlosung die AMF enthielt, als Ausgangspunkt zur Mykorrhizierung von Pflanzenwurzeln dienen kann. Die unterschiedlichen ökologischen Regenwurmgruppen unterschieden sich nicht in ihrem Einfluss auf die Biomasseproduktion der

Pflanzen und die Verbreitung von AMF. Diese Ergebnisse zeigen zum ersten Mal, dass die oft vernachlässigte unterirdisch abgelegte Regenwurmlosung einen großen Einfluss auf die Strukturvon Pflanzengemeinschaften haben, da sie unterschiedliche funktionelle Pflanzengruppen unterschiedlich beeinflussen.

8.3 Lebenslauf

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BERUFSPRAXIS

- Seit 01/2009 Wissenschaftliche Mitarbeiterin bei Bio Forschung Austria
- 09 & 10/ 2008 Praktikantin bei Bio Forschung Austria

PUPLIKATIONEN

Katharina Wechselberger, Johann G. Zaller, Wolfgang Wanek, Thomas Drapela, Markus Gorfer, Marcel van der Heijden, Thomas Frank (2009). Do earthworms affect the mycorrhizal colonisation of grassland plants? Poster presentation at the GFÖ 2009 (C2.P-6), Bayreuth , Germany.