

Earthworms promote greater richness and abundance in the emergence of plant species across a grassland-forest ecotone

Julia Clause^{1,2,*}, Sébastien Barot³ and Estelle Forey¹

¹ Ecodiv URA/EA-1293, Normandie Université, Université de Rouen, IRSTEA, SFR Scale 4116, UFR Sciences et Techniques, 76821 Mont Saint Aignan Cedex, France

² Centre de Formation sur l'Environnement et la Société (CERES), Ecole Normale Supérieure, 24 Rue Lhomond, 75231 Paris Cedex 5, France.

³ IRD – iEES Paris, 7, quai St Bernard, 75230 Paris Cedex 05, France

*Correspondence address. Ecodiv URA/EA-1293, Normandie Université, Université de Rouen, IRSTEA, SFR Scale 4116, UFR Sciences et Techniques, 76821 Mont Saint Aignan Cedex, France. Tel: +33(0)6-36-75-84-56; E-mail: juliamclause@gmail.com

Abstract

Aims

Chalk grasslands are subject to vegetation dynamics that range from species-rich open grasslands to tall and encroached grasslands, and woods and forests. In grasslands, earthworms impact plant communities and ecosystem functioning through the modification of soil physical, chemical and microbiological properties, but also through their selective ingestion and vertical transportation of seeds from the soil seed bank. Laboratory experiments showed that seed–earthworm interactions are species specific, but little is known on the impact of seed–earthworm interactions in the field. The overall aim of this study was to better understand seed–earthworm interactions and their impact on the plant community. First we analyzed the composition of seedlings emerging from casts after earthworm ingestion. Then we compared seedling composition in casts to the plant composition of emerging seedlings from the soil and of the aboveground vegetation along four stages of the secondary succession of chalk grasslands.

Methods

Four stages of the secondary succession of a chalk grassland—from open sward to woods—were sampled in Upper Normandy, France, in February 2010. Within each successional stage (×3 replicates), we sampled the standing vegetation, soil seed bank at three soil depths (0–2, 2–5 and 5–10 cm) and earthworm surface casts along transects. Soil and cast samples were water sieved before samples were spread onto trays and placed into a greenhouse. Emerging seedlings were counted and identified. Effect of successional stage

and origin of samples on mean and variability of abundance and species richness of seedlings emerging from casts and soil seed banks were analyzed. Plant compositions were compared between all sample types. We used generalized mixed-effect models and a distance-based redundancy multivariate analysis.

Important Findings

Seedling abundance was always higher in earthworm casts than in the soil seed bank and increased up to 5-fold, 4-fold and 3.5-fold, respectively, in the tall grassland, woods and encroached grassland compared to the soil surface layer. Species richness was also higher in earthworm casts than in the soil seed bank in all successional stages, with a 4-fold increase in the encroached grassland. The plant composition of the standing vegetation was more similar to that of seedlings from casts than to that of seedlings from the soil seed bank. Seedlings diversity emerging from casts in the tall and encroached grasslands tended toward the diversity found in woods. Our results indicate that earthworms may promote the emergence of seedlings. We also suggest that the loss of some plant species in the seed bank and the tall grass vegetation in intermediary successional stages modify the local conditions and prevent the further establishment of early-successional plant species.

Keywords: aboveground–belowground interactions, earthworm casts, seedling emergence, secondary succession, seed bank

Received: 2 April 2015, Revised: 22 December 2015, Accepted: 2 February 2016

INTRODUCTION

Seed banks play a major role in the dynamics and composition of plant communities (Bakker *et al.* 1996; Bossuyt and Honnay 2008; Fenner 2000; Luzuriaga *et al.* 2005). They constitute reserves of non-germinated seeds in the soil or at the soil surface (Csontos 2007). The viability of these seeds depends on seed characteristics as well as on external factors such as light, moisture or temperature (Benech-Arnold *et al.* 2000; Thompson and Grime 1979). Transient seed banks contain seeds that are germinable for less than a year, whereas seeds from persistent seed banks remain viable for more than a year, up to decades or longer (Thompson and Grime 1979). Seed longevity in the soil is particularly dependent on their size, shape and depth (Bekker *et al.* 1998; Thompson *et al.* 1993). Small spherical seeds that are located deep in the soil tend to live longer than large seeds in the soil surface layers (Bekker *et al.* 1998). The capacity of seeds to remain viable in the soil in a dormant state enables them to survive extreme events such as fire or drought (Thompson 2000) and to germinate under favorable conditions for seedling establishment.

Seed survival can be impacted by their ingestion by diverse organisms. Provided that seeds are not fully digested, seed ingestion may also lead to seed dispersal (endozoochory) and seedling establishment by triggering seed germination and by reducing seed dormancy (Janzen 1969; Traveset 1998). On the contrary, seed survival can also decrease when seeds are digested or severely damaged. Apart from the observed endozoochory in primates (Norconk and Veres 2011), grazing mammals (Neto *et al.* 1987) and birds (Barnea *et al.* 1991; but see Traveset 1998 for a complete review), seed ingestion by invertebrates has also been observed (Darwin 1881; Decaëns *et al.* 2003; Grant 1983; Vega *et al.* 2011). However, the number of studies in nature is still limited. Among invertebrates, earthworms have been subjects of recent attention (Clause *et al.* 2011; Decaëns *et al.* 2003; Eisenhauer *et al.* 2009a, 2009b, 2010).

Several studies showed the impacts of seed ingestion, digestion and egestion of seeds by earthworms on seed bank and plant communities (Eisenhauer *et al.* 2009b; McRill and Sagar 1973; Willems and Huijsmans 1994; see Forey *et al.* 2011 for a review). Seed ingestion, in association with earthworm movements, leads to the vertical transportation of seeds, i.e. their burial or surface exposure (Donath and Eckstein 2012; Willems and Huijsmans 1994; Zaller and Saxler 2007). In tropical grasslands, earthworm casts contain a higher seed density of viable seeds than the surrounding soil (Decaëns *et al.* 2003). Seeds surviving the digestion process are thought to benefit from a partial damage of their seed coat, which favors seed germination and seedling establishment (Ayanlaja *et al.* 2001; Eisenhauer *et al.* 2009a; McRill and Sagar 1973). Increased germination and seedling establishment might be further enhanced by cast properties. Specifically, casts tend to have a higher content in mineral nutrients and have particular physical and microbial properties (Clause *et al.* 2014;

Jouquet *et al.* 2008; Shipitalo and Protz 1989). Hence, due to favorable growth conditions associated with high numbers of viable seeds, casts are potentially important regeneration niches for some plant species (see Decaëns *et al.* 2003; Milcu *et al.* 2006). Additionally, earthworms selectively ingest seeds according to their size, shape, texture or oil content (Clause *et al.* 2011; Eisenhauer *et al.* 2009a; Janzen 1969; Regnier *et al.* 2008; Willems and Huijsmans 1994). Some studies suggest that earthworms prefer small seeds (Clause *et al.* 2011; Eisenhauer *et al.* 2009a) while others suggest the contrary (Regnier *et al.* 2008), and that they prefer non-grass seeds (either non-leguminous or leguminous) to grass seeds (Zaller and Saxler 2007).

Mechanisms behind the impact of seed–earthworm interactions via seed ingestion on plant communities are still unclear and few studies have focused on them in a natural context (Decaëns *et al.* 2003; Eisenhauer *et al.* 2009b; Willems and Huijsmans 1994). The importance of these interactions still needs to be assessed. The relatively undisturbed nature of species-rich semi-natural chalk grasslands constitutes an opportunity for studying the direct relationship between earthworms, seed banks and aboveground communities via seed ingestion, egestion in casts and the impact on seedling emergence. As earthworm communities vary along the grassland succession (Decaëns *et al.* 1998), these earthworm–seed relationships are likely to vary along a gradient of secondary succession.

Thus, we aimed at better understanding seed–earthworm interactions and their impact on plant communities across a grassland-forest ecotone. To do so, we analyzed the composition of seedlings emerging from casts after earthworm ingestion and compared it to the plant compositions of emerging seedlings from the soil and of the aboveground vegetation, along four stages of the secondary succession of chalk grasslands.

Two questions led our study: (i) do seeds preferentially germinate from earthworm casts than from the surrounding soil? and (ii) are assemblages of species germinating from earthworm casts similar to those found in the soil seed bank and the standing vegetation along the successional gradient? Overall, we discuss the potential of earthworms as drivers of the plant community assemblage.

MATERIALS AND METHODS

Study site

The study site is the natural reserve of Saint-Adrien (1°7'30"E, 49°22'22"N) located 15 km south of Rouen (Upper Normandy, France). Yearly average rainfalls and temperatures are 800 mm and 10°C, respectively. This 32 ha site is particularly well documented (Alard *et al.* 1998; Dutoit and Alard 1995; Dutoit *et al.* 2004). It is composed of a mosaic of different stages of secondary succession from open grasslands to scrubs and woods. Soils are shallow rendzinas (Rendzina, Protorendzina) under grassland communities and deeper

rendzinas (Brown Rendzina, Drift Brown Rendzina, Brown Calcareous soil) under shrubs and woods (Dutoit et al. 2004).

Four different successional stages of chalk grasslands were sampled to observe the temporal evolution of seed bank–earthworms interactions: open (O), tall (T) and encroached (E) grasslands and woods (W). Open grasslands are characterized by species-rich herbaceous vegetation dominated by *Carex flacca*, *Festuca lemanii* and *Teucrium chamaedrys*. Tall grasslands (T) are dominated by a grass species: *Brachypodium pinnatum* and are subject to summer mowing by Prim Holstein cows (3 ind.ha⁻¹). Encroached grasslands (E) are also dominated by *B. pinnatum* and are encroached with many shrub and ligneous species such as *Cornus sanguinea*, *Crataegus monogyna* and *Rosa canina*. The last stage (W) corresponds to an early forest dominated by maple trees (*Acer campestre*), common dogwoods (*C. sanguinea*) and common spindle (*Euonymus europaeus*).

Regarding earthworm composition, Decaëns et al. (1998) showed that endogeic species dominated in all successional stages. They also showed that density and biomass of anecic species increased in tall and encroached grasslands and those of epigeic species increased in woods (see Supplementary Table S1). Dominant endogeic species are *Allolobophora chlorotica* (Savigny) and *Aporrectodea caliginosa* (Savigny); dominant anecic species are *Lumbricus terrestris* (L.) and *Ap. giardi* (Savigny); dominant epigeic species are *Dendrodrilus rubida* (Savigny) and *Lumbricus rubellus* (Hoffmeister) (see Decaëns et al. 2008). Field observations suggest that the relative proportions of ecological groups of earthworm did not significantly change from data by Decaëns et al. (1998) within each successional stage.

Within each of the four successional stages, three 10-m transects were positioned perpendicularly to the slope and were spaced of at least 100 m. Five plots were chosen on each transect. In each plot, casts, soil seed bank and vegetation were sampled. In total, we gathered 300 samples = 3 transects × 4 successional stages × 5 plots × 5 sample origins (i.e. vegetation, casts and 3 soil depths). All transects were located at the center of each successional stage and at least 3 m from any other stage (see Luczaj and Sadowska 1997 for vascular plants) to avoid any edge effect. They were exposed to similar light and temperature conditions (South oriented).

Seedling emergence from soil and cast seed banks

The persistent soil seed bank was sampled in February 2010, when species with transient seed banks (Types I *sensu*, Thompson and Grime 1979) are not abundant in chalk grassland soils (Davies and Waite 1998). In each plot, four soil subsamples were collected with a soil core (ø 5 cm, every 50 cm). Each soil sample was separated into three depths (Gross 1990): 0–2, 2–5 and 5–10 cm. Subsamples for the four soil cores were pooled to obtain one sample per plot and per depth. The total mean volume sampled per transect was 5967.9 cm³, which is >1200 cm³, the volume needed to describe grassland seed banks (Roberts 1981).

Cast sampling was carried out in February 2010. In each plot, earthworm surface casts were manually collected in a 2 × 2 m quadrat (one person, 20 min/quadrat). This time period was chosen to sample a sufficient amount of cast while maintaining a constant sampling effort. Casts were easier to sample under low vegetation density with high density of casts (i.e. woods) than under tall vegetation (i.e. tall and encroached grasslands) or low cast density (open grassland). No distinction was made between casts of different earthworm species. Volume of casts sampled was: 75 ± 80 cm³ in open grasslands, 81 ± 45 cm³ in tall grasslands, 67 ± 36 cm³ in encroached grasslands and 114 ± 46 cm³ in woods.

All soil and cast samples were kept in the fridge for 2 weeks (5°C) to help break seed dormancy (Gross 1990), after their volume was measured in a beaker after removing coarse gravels from samples. Samples were then water sieved at 4 mm to remove the coarsest plant fragment and very fine gravels and at 0.2 mm to reduce soil volume (Ter Heerdt et al. 1996). We followed Ter Heerdt et al.'s (1996) germination approach to monitor seed bank species content. Although the total seed content is best assessed by the extraction method (see Weiterová 2008), it is labor-intensive and time-consuming. As our goal was to describe the impact of earthworms on overall chalk grassland plant communities, monitoring germinating seeds with this germination approach was sufficient.

All sieved samples were spread over a layer of moist gauze added to 3 cm vermiculite in a 34 × 61 cm tray. All trays were placed in a non-heated greenhouse for germination, and samples were watered regularly to keep optimal moisture levels. Trays were regularly randomly moved. Species were identified at the seedling stage with Muller's seedling determination key (Muller 1978) and counted before they were removed from the sample. Seedlings were then eliminated. Seedlings that could not be identified were grown further until identification was possible. After the first 2 months, samples were carefully turned over in order to facilitate the emergence of new seedlings. Seedlings that died during the experimentation and could not be identified were only added to the density data (26% of the total density).

Sampling of standing vegetation

The *in situ* aboveground vegetation (vascular plants) was sampled in June 2010 in each plot, i.e. five 2 × 2 m quadrats in each transect. The cover-abundance index of Braun-Blanquet (1964) was used to quantify the expressed vegetation: (i) cover < 5%; (ii) 5% < cover < 25%; (iii) 25% < cover < 50%; (iv) 50% < cover < 75%; (v) cover > 75%. The '+' code was used for species represented only by a few individuals. Species were identified with the nomenclature of Provost (1998).

Data analysis

A generalized linear mixed modeling (GLMM) approach was used to test the effect of the origin of samples (OS: standing vegetation, cast or soil layers a, b and c), the successional stage (S: O, T, E, W) and their interaction on the abundance and

species richness of germinating seedlings ($n = 15$). Transect identity was considered as a random effect to avoid pseudo-replication (Bolker *et al.* 2009). Seedling abundance and species richness were treated as count data. The volume of samples differed between sample origins and between stages. This difference of volume likely influenced their seed content in a non-linear manner. Therefore, an 'offset' term was used to integrate the volume of samples as covariate in our models (see Zuur *et al.* 2009). This volume of samples was log-transformed to improve normality. Species richness is very sensitive to the abundance of collected seeds. Not taking the abundance of seeds into account while performing the analysis might strengthen the effect of other factors and create a bias in data interpretation. Gotelli and Colwell (2011) suggest different ways to deal with this bias, among which treating species abundance as covariate. Therefore, the square-root-transformed seedling abundance was added as a covariate in the model testing the response of species richness. All response variables were best modeled with a Poisson distribution. Observation-level random effects (olre), where each data point receives a unique level of a random effect, were used to cope with overdispersion in count data when necessary (Harrison 2014). Final formulas for both models were: Abundance model = abundance $\sim S \times OS + \text{offset}(\text{Log}(\text{volume of sample})) + \text{random}(\text{transect identity}) + \text{random}(\text{olre})$ and Richness model = richness $\sim S \times OS + \text{sqrt}(\text{abundance}) + \text{offset}(\text{Log}(\text{volume of sample})) + \text{random}(\text{transect identity})$. Multiple comparisons tests were performed with Tukey's honest significance test (HSD) tests with *glht* in R that allows for Tukey's HSD comparisons of groups in mixed-effects models. Graphs were drawn with the 'effects' R package (Fox 2003).

To test for the contribution of successional stages and sample origins (cast, soil layers a, b and c, and vegetation) and their interaction on plant composition, we performed a distance-based redundancy analysis (db-RDA) according to Legendre and Anderson (1999), based on Bray-Curtis distance matrices. The effect of each variable was tested with a Monte-Carlo permutation test (n . permutations = 9999). Differences of plant composition among factors were observed on the associated graph. In order to compare vegetation and seed bank data, the Braun-Banquet indices of vegetation were converted into cover percentages using the median value of cover: 0.025 for the class '1' (cover < 5%); 0.15 for the class '2' (5% < cover < 25%); 0.375 for the class '3' (25% < cover < 50%); 0.625 for the class '4' (50% < cover < 75%); 0.875 for the class '5' (cover > 75%) and 0.0125 for the class '+'. For seed banks, species frequencies were calculated as the number of seedlings of each species divided by the total number of seedlings in the seed banks of each sample. Singletons and sites where no species emerged were removed from the analysis as Bray-Curtis indices cannot be calculated using '0' values, which resulted in a 279 (rows) by 108 (columns) matrix.

All analyses were performed with the 'R' statistical and programming environment (R Development Core Team 2013)

including the following packages: 'ade4' (Dray and Dufour 2007), 'lme4' (Bates *et al.* 2014), 'effects' (Fox 2003) and 'multcomp' (Hothorn *et al.* 2013) for the GLMM and LMM and 'vegan' (Oksanen *et al.* 2013) for the db-RDA.

RESULTS

Differences in seedling abundance and species richness between soil and casts

Totally, 3701 seedlings from 57 species were observed in the soil and cast seed banks (51 species in soil and 44 in casts). Four species—*Plantago media*, *Polygonum aviculare*, *Ranunculus repens*, *Thesium humifusum*—were found in the soil seed bank only and *Avenula pratensis* was found in the cast seed bank only (Supplementary Tables S2 and S3). *C. flacca* was the dominant species in the cast and in the soil seed banks, where it represented 35.4, 43.6, 56.3 and 61.4% of seedlings in the casts and soil layers a, b and c, respectively. *C. flacca*, *F. lemanii* and *C. monogyna* constituted 50.8% of seedlings emerging in the cast seed bank. *B. pinnatum*, *C. sanguinea*, *Sesleria albicans*, *C. flacca*, *T. chamaedrys*, *Genista tinctoria*, *Anthericum ramosum* and *F. lemanii* constituted 52.9% of the total aboveground vegetation cover.

The interaction between the successional stage and the OS influenced seedling abundance (GLMM: $\chi^2(18) = 186.06$, $P < 0.001$; Fig. 1a; Table 1). Seedling abundance was significantly higher in casts than in all soil samples in all stages of the secondary succession except in open grasslands (Fig. 1a; Supplementary Table S4). Seedling abundance in casts compared to the soil surface layer increased 5-fold in tall grasslands, 4-fold in woods and 3.5-fold in encroached grasslands (Fig. 1a; Supplementary Table S4). The 2-fold difference was not significant in open grasslands. Overall and in casts, seedling abundance was higher in tall and encroached grasslands than in the two other successional stages (GLMM: $\chi^2(3) = 18.62$, $P = 0.0003$ within casts). Seedling abundance was the lowest in woods (Fig. 1a).

Species richness of emerging seedlings was significantly influenced by the abundance of emerging seedlings in samples and by an interaction between the successional stage and the OS ($P < 0.001$ and $P = 0.007$, respectively; GLMM: $\chi^2(16) = 400.53$, $P < 0.001$; Table 1). Species richness was much higher in casts than in all soil samples in all stages of the secondary succession (Fig. 1b; Supplementary Table S4). Species richness was four times higher in casts than in the soil surface layer in encroached grasslands (Fig. 1b; Supplementary Table S4). It was also more than three times higher in casts in tall grasslands and twice in open grasslands (Fig. 1b; Supplementary Table S4). Overall, species richness was higher in all samples in tall and encroached grasslands than in the two other successional stages. Casts alone followed the same pattern but the difference was only significant between casts of encroached grasslands and of woods (Fig. 1b; Supplementary Table S4). Species richness decreased with soil sample depth.

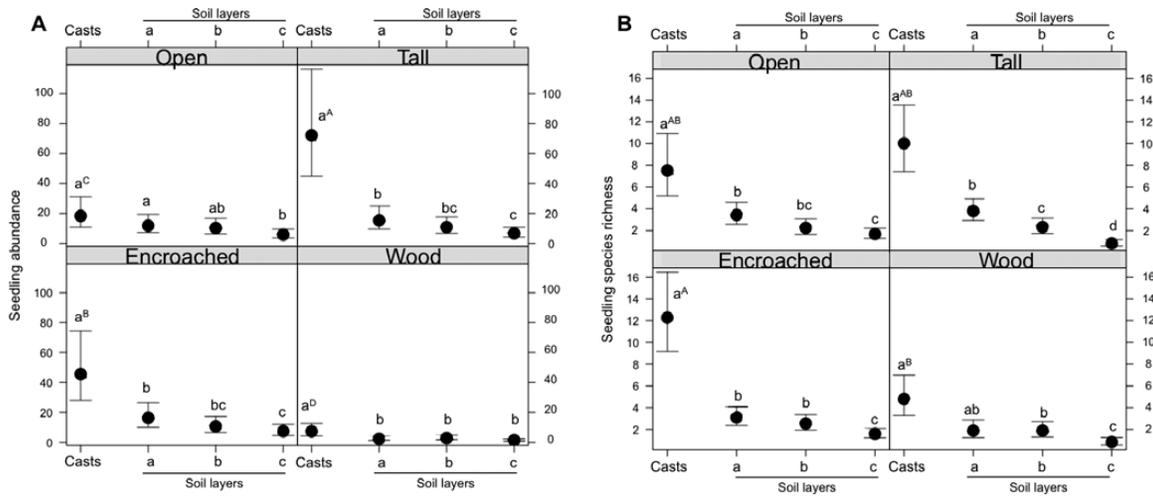


Figure 1: seedling abundance (A) and seedling species richness (B) in soil (Soil layers a, b, c) and cast (Casts) seed banks along the secondary succession. This graph shows mean effects (\pm SE) of the stage \times samples of different origins. Different lowercase letters indicate significant differences between samples within stages. Different capital letters in superscript indicate significant differences between casts of different stages (Tukey’s HSD, $\alpha = 0.05$).

Table 1: abundances and species richness of seed bank communities differ between soil samples and along successional stages

	Null model	Selected model			
		Formula ^a	AIC	df	χ^2
Seedling abundance	1773	S \times OS	1617	18	186.06 ^b
Species richness	1304	S \times OS + $\sqrt{\text{Abundance}}$	936	16	400.53 ^b

Akaike information criterion (AIC) values and associated residual degrees of freedom (df) are shown for the GLMM assessing the variation in seed abundance and plant species richness of emerged seedlings among successional stage (S), origin of samples (OS: cast, soil layers a, b and c) and their interaction.

^aOffsets and olre are not indicated.

^bLevels of significance with $\alpha < 0.001$ of models compared to a null model with no factor (null model = 1 + offset (Log(volume of sample) + random (transect identity) + random (olre)).

Effects of sample origin and successional gradient on plant composition from standing vegetation, soil seed bank and cast samples

The db-RDA showed that the OS contributed more to the similarity of plant composition than the stage of the secondary succession, and that both factors interacted significantly ($F_{(12,259)} = 2.4$, $P < 0.001$; Table 2).

The graphical representation of the db-RDA showed that plant composition varied between sample origins (Fig. 2 and Table 2). The vegetation samples were discriminated from the rest of the samples along the axis 1 (10.0 % of total inertia, $P < 0.001$; Fig. 2). Plant composition was closer between casts and the soil surface layer than between casts and the other soil layers b and c. Plant composition in casts was overall closer to the plant composition in the standing vegetation than the soil seed bank was (Fig. 2). Axis 1 was strongly influenced by the abundance of

Table 2: effect of successional stage, sample origin and their interaction on the plant composition of emerged seedlings and of the standing vegetation

	df	Inertia	% constrained inertia
Total		410.8	
Stage (S)	3	23.4	23.2 ^a
Origin of samples (OS)	4	43.2	42.7 ^a
S \times OS	12	34.3	34.0 ^a
Residuals	250	309.9	

Contributions of each factor are indicated as a percentage of the constrained inertia (=variance) and resulted from the Monte-Carlo permutation test ($n = 9999$) on the db-RDA. df, degrees of freedom.

^aLevels of significance with $\alpha < 0.001$.

B. pinnatum in the vegetation of all grasslands and the abundance of *C. flacca* in the vegetation of open and tall grasslands.

Differences of plant composition varied along the secondary succession (Fig. 2 and Table 2). Samples from woods were discriminated from the other stages along the axis 2 (3.3% of total inertia, $P < 0.001$). The difference between sample origins was always higher in the tall grasslands than in encroached grasslands, followed by open grasslands and woods (Fig. 2). In open grassland, the plant composition did not differ between casts and any soil layer. It differed between casts and soil layers b and c in all other successional stages. Plant composition in casts in tall and encroached grasslands shifted towards the plant composition of casts and of vegetation in woods (Fig. 2).

DISCUSSION

Higher seedling abundance and species richness in casts than in the soil seed bank

In all stages of the secondary succession, more seedlings of more species emerged from casts than from the soil seed bank.

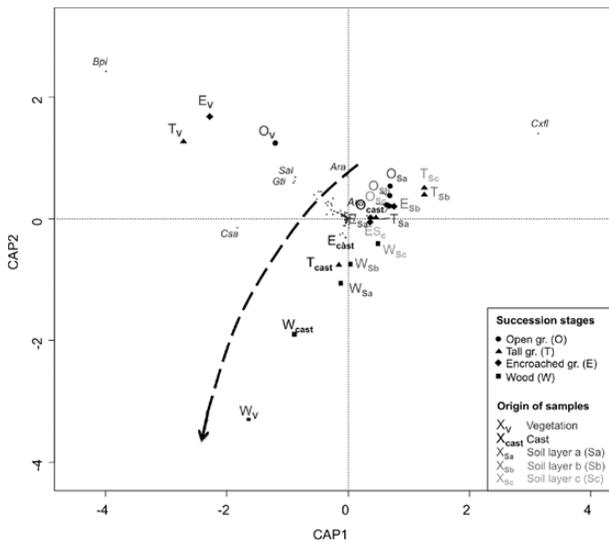


Figure 2: characterization of plant composition depending on sample types and stages of succession using a db-RDA based on Bray-Curtis distances (CAP1 = 10.0%; CAP2 = 3.3% of total variance). Species frequencies were used for seed banks and median of cover percentage was used for the standing vegetation. Different symbols indicate different succession stages. Color gradient indicates different origins of samples (see legend). Six selected dominant species were indicated: *Anthericum ramosum* (Ara), *Brachypodium pinnatum* (Bpi), *Carex flacca* (Cxfl), *Cornus sanguinea* (Csa), *Genista tinctoria* (Gti), *Sesleria albicans* (Sal). The arrow indicates a trend for the acceleration of the succession with cast seedling composition.

Although only emerging seedlings were taken into account with Ter Heerdt *et al.*'s (1996) method, we suggest that this higher seedling abundance in casts could reflect higher seed content in casts. Earthworms actively select seeds, and actively or passively ingest them, thereby leading to a higher seed density and seedling emergence in casts. The aggregation of seeds in casts supports previous results in grasslands, forests and croplands (Clause *et al.* 2015; Decaëns *et al.* 2003; Regnier *et al.* 2008; Smith *et al.* 2005; Willems and Huijsmans 1994).

A higher seedling abundance in casts could also be explained by a higher germination rate of seeds emerging from the cast due to the passage of seeds through the earthworm gut. The abrasion of the seed coat within the earthworm gizzard has been shown to break physical seed dormancy (McRill and Sagar 1973; Venier *et al.* 2012). Enzymatic activity of earthworms and microorganisms within the earthworm gut could also have altered the seed coat (Fujii *et al.* 2012; Lattaud *et al.* 1998). Other studies showed a decreased seed germination after the earthworm gut passage (Eisenhauer *et al.* 2009a), or their death via a total digestion of seeds such as that of *Origanum vulgare* and *Urtica dioica* by *L. terrestris* (Clause 2014). Decaëns *et al.* (2003) showed that earthworm casts in Colombian grasslands contained more seeds than the surrounding soil. These seeds were also less viable. Therefore, we suggest that earthworms do aggregate seeds in their casts, but that their specific selection and ingestion of seeds might not

necessarily lead to the guaranteed germination of all of them. To distinguish the specific impact of seed ingestion on germination and emergence at the field scale, it would be complementary to visually sort and count seeds in the soil and casts before applying the germination protocol.

We found a higher species richness in casts than in the surrounding soil in all stages. This result that takes the sample volume of casts and soil samples, and the seedling abundance into account (see Materials and Methods) suggests that many seeds survived gut passage. A higher seed abundance in casts partially explained this higher species richness. Several *in situ* and experimental studies showed a specific seed selection by earthworms (Aira and Pearce 2009; Asshoff *et al.* 2010; Clause *et al.* 2011; Eisenhauer and Scheu 2008a; Eisenhauer *et al.* 2009a, 2009b; McRill and Sagar 1973; Milcu *et al.* 2006). Thus, we expected the richness of seedling species emerging from casts to be lower than the surrounding soil due to a seed selection. On the other hand, vertical movements of earthworms may increase the probability for earthworms to encounter a higher species richness of seeds.

The high richness of seeds in earthworm casts in our results can also be explained by the diversity of earthworm species in chalk grasslands of Upper Normandy (Decaëns *et al.* 1998; Margerie *et al.* 2001; Supplementary Table S1). Seed–earthworm interactions do not only depend on seed species, but also on earthworm species that may select specific seed species (Asshoff *et al.* 2010; Clause *et al.* 2011; Eisenhauer *et al.* 2009a). The higher seedling abundance and species richness was found in tall and encroached grasslands, which were associated with a higher density of anecic earthworms than in other successional stages (Supplementary Table S1; Decaëns *et al.* 1998). Anecic earthworms ingest soil and produce a large quantity of casts at the soil surface due to their vertical movement (Lee 1985). They also ingest a larger range of seed sizes than other ecological groups due to their bigger size, although this result varies among studies (Clause *et al.* 2011; Eisenhauer *et al.* 2009a). When they cannot directly ingest seeds, they gather them in their middens—small mounds of casts—at the soil surface for further consumption (Eisenhauer and Scheu 2008b). The distinction between casts of each earthworm species is difficult and differences in the proportions of anecic, endogeic and epigeic earthworms (Supplementary Table S1) likely explained the heterogeneity of ingestion patterns. Therefore, although microcosm studies showed a high selective seed ingestion by earthworms (Asshoff *et al.* 2010; Clause *et al.* 2011; Eisenhauer *et al.* 2009a, 2009b; McRill and Sagar 1973), our *in situ* patterns show a diluted selection and effect on seeds.

Variations of plant composition along the succession and potential impact of earthworms

We found that the plant composition was more similar between casts and the standing vegetation than between vegetation and the soil surface layer a, suggesting that earthworms contribute to the emergence of plants in the

vegetation. However, the discrimination of the standing vegetation compared to that of the casts and the soil seed banks in all grasslands in the db-RDA suggests that seedlings that emerged from the seed bank in the greenhouse fail to emerge in the field. This is likely due to the vegetative reproduction of the abundant grass species sampled in the vegetation (*B. pinnatum*, *F. lemanii*, *Carex* spp. and *Briza media*), the first being described as a social species due to its high ability to expand mostly by vegetative means and dominate species-poor tall grassland successional stage. The abundance and the height of these grasses reduce light penetration to the ground and may limit the germination of seeds whose light requirements are not fulfilled (Saar et al. 2012). This impact of a tall vegetation was supported by the high difference of plant composition between sample origins along the secondary succession. Plant composition between sample origins differed more in the tall grassland than in the three other successional stages, especially in the open grassland and woods.

The graphical representation of the db-RDA showed that the ingestion and egestion of seeds by earthworms contributed to a differentiation of plant composition along the secondary succession of chalk grasslands. The composition of seedlings that emerge from casts tends towards the vegetation of the last successional stage, i.e. woods. Mulrák et al. (2012) showed that earthworms drove the succession of plant communities in an *in situ* experimental study in post-mining sites. We suggest that the higher similarity of plant composition between sample origins in woods than in grasslands was due to the physical barrier created by trees that prevented the seed rain to reach the wood soil. In those conditions, pools of seeds in woods were not renewed and species were lost when seeds became unviable. The fragmentation of habitats such as grasslands leads indeed to a high extinction rate of species (Saar et al. 2012) and to the loss of grassland seeds species from transient or short-term persistent seed banks (Bakker et al. 1996; Bekker et al. 1998; Bossuyt et al. 2006; Thompson 2000). Other studies showed that the seed bank and the vegetation were the most similar in grasslands and the least similar in forests (Bossuyt and Honnay 2008; Bossuyt et al. 2006; Hopfensperger 2007; Jacquemyn et al. 2011). Thus, we suggest that the high similarity of plant composition between samples of different origins in woods was mostly explained by an absence of similar species in those samples. Surprisingly, earthworm activity uncovered seeds of grassland species such as *C. flacca*, *Daucus carota*, *F. lemanii*, *Hieracium pilosella* and *Linum catharticum* that should rather reduce the similarity between the vegetation and the cast seed bank (Supplementary Tables S2 and S3). This aspect was obscured by the abundance of the seeds of *C. sanguinea* found in the casts. The presence of seeds of those five species from transient or persistent seed banks (see Thompson et al. 1993) suggests that the physical structure of casts could also play a protective role from environmental conditions and could prevent seed germination (Jouquet et al. 2008; Schrader and Zhang 1997). Although the overall viability of seeds seems to be lower in casts and to differ among seed species due to the passage through the earthworm gut (Decaëns

et al. 2003; McRill and Sagar 1973), no study has been done to study the impact of cast age on seed viability. The stimulation of specific microbial and fungal populations and enzyme activities in fresh casts and their temporal variation (Tiwari et al. 1989; Tiwari and Mishra 1993) or the further deterioration of seeds by microbial communities (Aira et al. 2005) might impact seed viability across time.

CONCLUSION

Few *in situ* studies have been achieved on the impact of seed–earthworm interaction on plant communities via seed ingestion and the emergence of seedlings. Our results support previous findings documenting the aggregation of seeds in casts. Earthworms did ingest a particular composition of seeds from the soil seed bank and had an impact on the emergence of specific seedling species. However, this pattern could not be attributed to specific earthworm species, and patterns were not consistent along the succession. Taken together and considering the number of seedling species emerging from casts, earthworms in our chalk grassland could be considered to have a quite generalist feeding behavior, which shows in the emergence patterns. The high species richness and the higher similarity between casts and vegetation than the similarity between soil and vegetation suggests that earthworms potentially promote the germination and establishment of some species of early chalk grassland succession, and thereby impact the grassland dynamics. The plant composition of standing vegetation differed from that of seedlings emerging from our seed bank in lab conditions. We suggest that the domination of grasses and tall plants in intermediary successional stages prevented a further establishment of species emerging from casts and from the soil seed bank. Further laboratory studies on seed–earthworm species-specific interactions are needed to determine the seeds that earthworms preferentially ingest and their impact on seedling performances. Long-term experimental field studies manipulating earthworm density could help clarify the impact of earthworms on the dynamics of plant communities in the chalk grassland, following the long-term mesocosm experiment of Laossi et al. (2011).

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Plant Ecology* online.

FUNDING

Upper Normandy region and the SCALE research network.

ACKNOWLEDGEMENTS

The authors would like to thank the Conservatoire Naturel de Haute-Normandie for allowing the access to the site. They would also like to particularly thank Pierre Margerie for his comments and recommendations

on site sampling and manuscript editing, and Philippe Delporte, Gaylord Dujardin, Marthe Akpa-Vincelas, Eléonore Rebuffat and Gauthier Lappa for technical and on site help. Finally, the authors thank two anonymous reviewers and editor-in-chief Cameron Wagg for their comments and thorough suggestions that significantly improved our manuscript.

Conflict of interest statement. None declared.

REFERENCES

- Aira M, Monroy F, Domínguez J (2005) Ageing effects on nitrogen dynamics and enzyme activities in casts of *Aporrectodea caliginosa* (Lumbricidae). *Pedobiologia* **49**:467–73.
- Aira M, Pearce TG (2009) The earthworm *Lumbricus terrestris* favours the establishment of *Lolium perenne* over *Agrostis capillaris* seedlings through seed consumption and burial. *Appl Soil Ecol* **41**:360–3.
- Alard D, Poudevigne I, Dutoit T, et al. (1998) Dynamique de la biodiversité dans un espace en mutation. Le cas des pelouses calcicoles de la basse vallée de Seine. *Acta Oecol* **19**:275–84.
- Asshoff R, Scheu S, Eisenhauer N (2010) Different earthworm ecological groups interactively impact seedling establishment. *Eur J Soil Biol* **46**:330–4.
- Ayanlaja SA, Owa SO, Adigun MO, et al. (2001) Leachate from earthworm castings breaks seed dormancy and preferentially promotes radicle growth in jute. *HortScience* **36**:143–4.
- Bakker J, Poschlod P, Strykstra R, et al. (1996) Seed banks and seed dispersal: important topics in restoration ecology. *Acta Bot Neerl* **45**:461–90.
- Barnea A, Yom-Tov Y, Friedman J (1991) Does ingestion by birds affect seed germination? *Funct Ecol* **5**:394–402.
- Bates D, Maechler M, Bolker BM, et al. (2014) lme4: Linear mixed-effects models using Eigen and S4. R package version 1.1–7. <http://CRAN.R-project.org/package=lme4> (January 2015, date last accessed).
- Bekker RM, Bakker JP, Grandin U, et al. (1998) Seed size, shape and vertical distribution in the soil: indicators of seed longevity. *Funct Ecol* **12**:834–42.
- Benech-Arnold RL, Sánchez RA, Forcella F, et al. (2000) Environmental control of dormancy in weed seed banks in soil. *Field Crop Res* **67**:105–22.
- Bolker BM, Brooks ME, Clark CJ, et al. (2009) Generalized linear mixed models: a practical guide for ecology and evolution. *Trends Ecol Evol* **24**:127–35.
- Bossuyt B, Honnay O (2008) Can the seed bank be used for ecological restoration? An overview of seed bank characteristics in European communities. *J Veg Sci* **19**:875–84.
- Bossuyt H, Six J, Hendrix PF (2006) Interactive effects of functionally different earthworm species on aggregation and incorporation and decomposition of newly added residue carbon. *Geoderma* **130**:14–25.
- Braun-Blanquet J (1964) *Pflanzensoziologie: Grundzüge der vegetationskunde*. Wien, New York: Springer.
- Clause J (2014) Seed earthworm relationships: seed selection and the responses of plant communities. *Ph.D. Thesis*. Université de Rouen.
- Clause J, Forey E, Lortie CJ, et al. (2015) Non-native earthworms promote plant invasion by ingesting seeds and modifying soil properties. *Acta Oecol* **64**:10–20.
- Clause J, Barot S, Richard B, et al. (2014) The interactions between soil type and earthworm species determine the properties of earthworm casts. *Appl Soil Ecol* **83**:149–58.
- Clause J, Margerie P, Langlois E, et al. (2011) Fat but slim: criteria of seed attractiveness for earthworms. *Pedobiologia* **54**:S159–65.
- Csontos P (2007) Seed banks: ecological definitions and sampling considerations. *Commun Ecol* **8**:75–85.
- Darwin CR (1881) *The Formation of Vegetable Mould, Through the Action of Worms, With Observations on Their Habits*. John Murray, London: Royaume-Uni.
- Davies A, Waite S (1998) The persistence of calcareous grassland species in the soil seed bank under developing and established scrub. *Plant Ecol* **136**:27–39.
- Decaëns T, Dutoit T, Alard D, et al. (1998) Factors influencing soil macrofaunal communities in post-pastoral successions of western France. *Appl Soil Ecol* **9**:361–7.
- Decaëns T, Margerie P, Aubert M, et al. (2008) Assembly rules within earthworm communities in North-Western France—a regional analysis. *Appl Soil Anal* **39**:321–35.
- Decaëns T, Mariani L, Betancourt N, et al. (2003) Seed dispersion by surface casting activities of earthworms in Colombian grasslands. *Acta Oecol* **24**:175–85.
- Donath TW, Eckstein RL (2012) Litter effects on seedling establishment interact with seed position and earthworm activity. *Plant Biol* **14**:163–70.
- Dray S, Dufour A (2007) The ade4 package: implementing the duality diagram for ecologists. *J Stat Softw* **22**:1–20.
- Dutoit T, Alard D (1995) Permanent seed banks in chalk grassland under various management regimes: their role in the restoration of species-rich plant communities. *Biodivers Conserv* **4**:939–50.
- Dutoit T, Buisson E, Roche P, et al. (2004) Land use history and botanical changes in the calcareous hillsides of Upper-Normandy (north-western France): new implications for their conservation management. *Biol Conserv* **115**:1–19.
- Eisenhauer N, Butenschoen O, Radsick S, et al. (2010) Earthworms as seedling predators: importance of seeds and seedlings for earthworm nutrition. *Soil Biol Biochem* **42**:1245–52.
- Eisenhauer N, Scheu S (2008a) Earthworms as drivers of the competition between grasses and legumes. *Soil Biol Biochem* **40**:2650–9.
- Eisenhauer N, Scheu S (2008b) Invasibility of experimental grassland communities: the role of earthworms, plant functional group identity and seed size. *Oikos* **117**:1026–36.
- Eisenhauer N, Schuy M, Butenschoen O, et al. (2009a) Direct and indirect effects of endogeic earthworms on plant seeds. *Pedobiologia* **52**:151–62.
- Eisenhauer N, Straube D, Johnson EA, et al. (2009b) Exotic ecosystem engineers change the emergence of plants from the seed bank of a deciduous forest. *Ecosystems* **12**:1008–16.
- Fenner M (2000) *Seeds: The Ecology of Regeneration in Plant Communities*. Wallingford, UK: CAB International.
- Forey E, Barot S, Decaëns T, et al. (2011) Importance of earthworm-seed interactions for the composition and structure of plant communities: a review. *Acta Oecol* **37**:594–603.
- Fox J (2003) Effect displays in R for generalised linear models. *J Stat Softw* **8**:1–27.
- Fujii K, Ikeda K, Yoshida S (2012) Isolation and characterization of aerobic microorganisms with cellulolytic activity in the gut of endogeic earthworms. *Int Microbiol* **15**:121–33.

- Gotelli NJ, Colwell RK (2011) Estimating species richness. In Magurran AE, McGill BJ (eds). *Frontiers in Measurement and Assessment*. New York: Oxford University Press, 39–54.
- Grant JD (1983) The activities of earthworms and the fates of seeds. In Satchell JE (ed). *Earthworm Ecology from Darwin to Vermiculture*. London: Chapman and Hall Ltd, 107–22.
- Gross KL (1990) A comparison of methods for estimating seed numbers in the soil. *J Ecol* **78**:1079–93.
- Harrison XA (2014) Using observation-level random effects to model overdispersion in count data in ecology and evolution. *PeerJ* **2**:e616.
- Hopfensperger KN (2007) A review of similarity between seed bank and standing vegetation across ecosystems. *Oikos* **116**:1438–48.
- Hothorn T, Bretz F, Westfall P, et al. (2013) multcomp: simultaneous inference in general parametric models. *Biometrical J* **50**:346–63.
- Jacquemyn H, Mechelen CV, Brys R, et al. (2011) Management effects on the vegetation and soil seed bank of calcareous grasslands: an 11-year experiment. *Biol Conserv* **144**:416–22.
- Janzen DH (1969) Seed-eaters versus seed size, number, toxicity and dispersal. *Evolution* **23**:1–27.
- Jouquet P, Bottinelli N, Podwojewski P, et al. (2008) Chemical and physical properties of earthworm casts as compared to bulk soil under a range of different land-use systems in Vietnam. *Geoderma* **146**:231–8.
- Laossi K-R, Noguera DC, Decaëns T, et al. (2011) The effects of earthworms on the demography of annual plant assemblages in a long-term mesocosm experiment. *Pedobiologia* **54**:127–32.
- Lattaud C, Locati S, Mora P, et al. (1998) The diversity of digestive systems in tropical geophagous earthworms. *Appl Soil Ecol* **9**:189–95.
- Lee KE (1985) *Earthworms: Their Ecology and Relationships With Soils and Land Use*. New York: Academic Press.
- Legendre P, Anderson MJ (1999) Distance-based redundancy analysis: testing multispecies responses in multifactorial ecological experiments. *Ecol Monogr* **69**:1–24.
- Łuczaj Ł, Sadowska B (1997) Edge effect in different groups of organisms: vascular plant, bryophyte and fungi species richness across a forest-grassland border. *Folia Geobot Phytotax* **32**:343–53.
- Luzuriaga AL, Escudero A, Olano JM, et al. (2005) Regenerative role of seed banks following an intense soil disturbance. *Acta Oecol* **27**:57–66.
- Margerie P, Decaëns T, Bureau F, et al. (2001) Spatial distribution of earthworm species assemblages in a chalky slope of the Seine Valley (Normandy, France). *Eur J Soil Biol* **37**:291–6.
- McRill M, Sagar GR (1973) Earthworms and seeds. *Nature* **243**:482.
- Milcu A, Schumacher J, Scheu S (2006) Earthworms (*Lumbricus terrestris*) affect plant seedling recruitment and microhabitat heterogeneity. *Funct Ecol* **20**:261–8.
- Muller FM (1978) *Seedlings of the North-Western European Lowland*. The Hague, The Netherlands: Dr. W Junk Publishers.
- Mulr ak O, Uteseny K, Frouz J (2012) Earthworms drive succession of both plant and Collembola communities in post-mining sites. *Appl Soil Ecol* **62**:170–7.
- Neto MS, Jones RM, Ratcliff D (1987) Recovery of pasture seed ingested by ruminants. 1. Seed of six tropical pasture species fed to cattle, sheep and goats. *Aust J Exp Agric* **27**:239–46.
- Norconk MA, Veres M (2011) Physical properties of fruit and seeds ingested by primate seed predators with emphasis on sakis and bearded sakis. *Anat Rec (Hoboken)* **294**:2092–111.
- Oksanen J, Blanchet FG, Kindt R, et al. (2013) vegan: Community Ecology Package. R package version 1.1–7. <http://CRAN.R-project.org/package=lme4> (June 2014, date last accessed)
- Provost M (1998) *Flore vasculaire de Basse-Normandie*. Caen, France: Presses universitaires de Caen.
- R Development Core Team (2013) *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.
- Regnier E, Harrison SK, Liu J, et al. (2008) Impact of an exotic earthworm on seed dispersal of an indigenous US weed. *J Appl Ecol* **45**:1621–9.
- Roberts HA (1981) Seed banks in soils. *Adv Appl Biol* **6**:1–55.
- Saar L, Takkis K, Pärtel M, et al. (2012) Which plant traits predict species loss in calcareous grasslands with extinction debt? *Divers Distrib* **18**:808–17.
- Schrader S, Zhang H (1997) Earthworm casting: stabilization or destabilization of soil structure? *Soil Biol Biochem* **29**:469–75.
- Shipitalo MJ, Protz R (1989) Chemistry and micromorphology of aggregation in earthworm casts. *Geoderma* **45**:357–74.
- Smith RG, Gross KL, Januchowski S (2005) Earthworms and weed seed distribution in annual crops. *Agric Ecosyst Environ* **108**:363–7.
- Ter Heerdt GNJ, Verweij GL, Bekker RM, et al. (1996) An improved method for seed bank analysis: seedling emergence after removing the soil by sieving. *Funct Ecol* **10**:144–51.
- Thompson K (2000) The functional ecology of soil seed banks. In Fenner M (ed). *Seeds: The Ecology of Regeneration in Plant Communities*. Wallingford, UK: CAB International, 215–35.
- Thompson K, Band SR, Hodgson JG (1993) Seed size and shape predict persistence in soil. *Funct Ecol* **7**:236–41.
- Thompson K, Grime JP (1979) Seasonal variation in the seed banks of herbaceous species in ten contrasting habitats. *J Ecol* **67**:893–921.
- Tiwari SC, Mishra RR (1993) Fungal abundance and diversity in earthworm casts and in uningested soil. *Biol Fertil Soils* **16**:131–4.
- Tiwari SC, Tiwari BK, Mishra, RR (1989) Microbial populations, enzyme activities and nitrogen-phosphorus-potassium enrichment in earthworm casts and in the surrounding soil of a pineapple plantation. *Biol Fertil Soils* **8**:178–82.
- Traveset A (1998) Effect of seed passage through vertebrate frugivores' guts on germination: a review. *Perspect Plant Ecol Evol Syst* **1**:151–90.
- Vega C, de Arista M, Ortiz PL, et al. (2011) Endozoochory by beetles: a novel seed dispersal mechanism. *Ann Bot* **107**:629–37.
- Venier P, Carrizo Garc a C, Cabido M, et al. (2012) Survival and germination of three hard-seeded *Acacia* species after simulated cattle ingestion: the importance of the seed coat structure. *S Afr J Bot* **79**:19–24.
- Weiterova I (2008) Seasonal and spatial variance of seed bank species composition in an oligotrophic wet meadow. *Flora* **203**:204–14.
- Willems JH, Huijsmans KGA (1994) Vertical seed dispersal by earthworms: a quantitative approach. *Ecography* **17**:124–30.
- Zaller JG, Saxler N (2007) Selective vertical seed transport by earthworms: implications for the diversity of grassland ecosystems. *Eur J Soil Biol* **43**:S86–91.
- Zuur A, Ieno EN, Walker N, et al. (2009) *Mixed Effects Models and Extensions in Ecology With R*. New York: Springer.