Management and inoculation strategies to promote anecic earthworm *Lumbricus terrestris* (L.) in grasslands on sandy soil

Harnessing native ecosystem engineers to improve water regulation for climate adaptation



ROOS VAN DE LOGT

Propositions

1. Earthworm populations in the field are more heterogenous in time and space than our sampling methods can capture.
   (this thesis)
2. Only part of the grasslands that are suitable for *Lumbricus terrestris* currently host a population.
   (this thesis)
3. AI improves the quality of PhD theses, but it decreases the quality of the PhD trajectory.
4. The scientific agenda is a nail in the coffin of true transition.
5. Animal food *production* systems are more adequately referred to as animal food *reduction* systems.
6. Outdoor play boosts the physical and mental well-being of adults.

Propositions belonging to the thesis, entitled

Management and inoculation strategies to promote anecic earthworm *Lumbricus terrestris* (L.) in grasslands on sandy soil

Roos van de Logt
Wageningen, 3 December 2026

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Management and inoculation strategies to promote anecic earthworm *Lumbricus terrestris* (L.) in grasslands on sandy soil

Harnessing native ecosystem engineers to improve water regulation for climate adaptation

Roos van de Logt

Thesis

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Summary

Agricultural grasslands deliver many different ecosystem services: not only do they provide feed for animals, they also play an important role in climate change mitigation through carbon storage and they support above- and below-ground biodiversity, cultural heritage and **water regulation**. As precipitation patterns are shifting due to climate change, the incidence of peak rains and prolonged droughts is rising, resulting in harvest losses, floods and infrastructural damage. The prevalence and intensity of such severe weather events is expected to increase in the future. As a consequence, the water-regulating capacity of soils, which is defined as **the capacity of soils to absorb, retain and supply water**, is of growing importance.

The water-regulating capacity of the soil is mainly determined by soil properties. While some of these properties are virtually unchanging, such as texture and mineralogy, others, such as structure and organic matter content, are influenced by plant-soil interactions, soil biota and agricultural practices such as soil cultivation. Some soils are more susceptible to peak rains and droughts than others. For example, the elevated sandy soils situated in the south and east of the Netherlands are vulnerable to both. The ± 4,500 dairy farmers as well as the water boards in these areas have been looking for ways to improve the water-regulating capacity of their soils. Soil management and soil biota, or, more specifically, **earthworms**, play an important role.

Research has shown that deep burrowing earthworms such as *Lumbricus terrestris* can contribute to an improved water infiltration rate and infiltration capacity. The species can thrive in grasslands, it is native to Europe and it can be found on all major soil types in the Netherlands; nevertheless, at the field-level, the species is not found everywhere and sometimes it occurs in very low population densities. There is much potential for improved water regulation if *L. terrestris* populations are stimulated or inoculated into grasslands on sandy soil. The aim of this thesis was to improve our knowledge about **habitat suitability** for *L. terrestris* in grassland on sandy soil and to explore the potential of *L. terrestris* **management and inoculation** with the ultimate goal of supporting climate adaptation in grasslands.

In **Chapter 2** we aimed to elucidate which characteristics in grasslands on sandy soil affect the presence and abundance of *L. terrestris*. A better understanding of the needs of the species helps to determine which grasslands could be suitable to host a population and which management practices could support and stimulate the abundance of *L. terrestris*. To this end, we carried out a field inventory on grasslands on sandy soil in the south of the Netherlands. We searched for correlations between

the presence and abundance of *L. terrestris* and soil texture, hydrology, interspecific competition with epigeic species *Lumbricus rubellus* and time passed since the last mechanical soil disturbance. It was found that *L. terrestris* was present under various textures, groundwater levels, species assemblies and grassland ages. Abundance showed a positive correlation with soil loaminess at 30-40 cm depth and a negative correlation with abundance of *L. rubellus*. No significant effects of groundwater level and grassland age were found.

In **Chapter 3**, we examined the effect of grassland management – more specifically grassland renewal – on earthworm population dynamics. Grassland renewal is an important but underexposed management measure. It is used to boost the productivity and improve the botanical composition of grasslands. The procedure consists of destroying the existing sward by herbicide application or tillage and then reseeding it, either directly or after one or multiple seasons of arable cropping. Data was collected in an on-farm mesocosm experiment, which was repeated for two consecutive years. We tested the effect of several grassland renewal strategies on the abundance, demography and species diversity of the earthworm community. These strategies included a combination of treatments with or without tillage and with or without vegetation cover loss. After six months we found a clear effect on earthworm abundance, which decreased along a gradient of increasing disturbance levels (low to high disturbance level: continued grassland without reseeding > no tillage with bare soil > tillage applied with direct reseeding > tillage applied with bare soil). Tillage had a stronger negative effect on earthworm abundance than soil cover loss, and their combined effects were most detrimental. Especially endogeic species (*Aporrectodea caliginosa* and *Aporrectodea rosea*) decreased in abundance when tillage was applied and when soil cover was lost. Generally, epigeics and anecics were less abundant and they were less clearly affected by the treatments. Adult earthworm abundance was more affected than juvenile abundance, six months after treatment application. Species diversity did not change.

In Chapters 4 and 5 we zoomed in on the inoculation of *L. terrestris* into grasslands on sandy soil. **Chapter 4** describes an on-farm mesocosm study in which *L. terrestris* was inoculated into grassland on two different farms. The grasslands had no resident *L. terrestris* population, but they seemed suitable for hosting the species, based on insights from literature and the inventory in Chapter 2. The total duration of the experiment was 15 months, and it was found that *L. terrestris* was able to survive, burrow to a depth of > 60 cm and produce offspring. Survival rate was 32% after seven months and 6% after 15 months. There were indications of interspecific competition between

L. terrestris and *L. rubellus* at the expense of *L. terrestris*. Several possible reasons that could explain why the *L. terrestris* survival rate was low were proposed: drought, as it was a dry summer; competition for food with *L. rubellus*; and low adaptability of the commercially purchased inoculum (imported from Canada) to the new environment.

A follow-up experiment was conducted in **Chapter 5**, in which we tested the hypotheses that had emerged from Chapters 2 and 4. A similar mesocosm setup was used, but with several adjustments. First, more food was provided to prevent competition with *L. rubellus*. Second, *L. terrestris* inoculum of two different origins were compared, locally collected and commercially purchased (imported from Canada). Third, *L. terrestris* was introduced into two different grasslands: one that already had a resident *L. terrestris* population, and another that did not. The grassland with a resident population was selected to enable us to separate the effects of inoculation from suitability of the habitat, and VIE tags were used to distinguish between inoculated and resident earthworms. The experiment lasted for 12 months. As in Chapter 4, *L. terrestris* survived, burrowed to > 60 cm deep and produced offspring. Survival rate was 15% at the farm with a resident population and 26% at the farm without. Local inoculum performed slightly better than Canadian inoculum.

The insights that emerged from Chapters 2-5 were integrated with each other and existing literature in **Chapter 6**. Additionally, I reviewed the conditions under which sandy soil can be a suitable habitat for *L. terrestris*. Then I discussed how severely disturbed earthworm populations managed to recover surprisingly well in some of the experiments. Lastly I explained how historical land-use and limited accessibility of grasslands could lead to the absence of *L. terrestris* in grasslands that could in fact offer a suitable habitat for the species. I also discussed the strengths and weaknesses of this study: non-destructive sampling methods for earthworms would further improve our monitoring ability, more data on soil moisture would be useful, and although mesocosms were suitable for testing *L. terrestris* inoculation as a proof of concept, the next step would be to set up long-term open-field experiments.

Lastly, I aimed to place this research in a broader perspective, emphasising the importance of a systems approach in the quest for climate adaptation. I discussed practical implications for researchers, farmers, earthworm breeders and water boards. They should bring together scientific expertise, a pragmatic framework, hands-on experience, a network and financial incentives to move the field forward by assessing feasibility in the field and developing successful management and inoculation strategies.



Samenvatting

Agrarisch grasland voorziet in vele verschillende ecosysteemdiensten: graslanden produceren voer voor dieren, maar leggen ook koolstof vast en dragen daarmee bij aan het voorkomen van klimaatverandering, ze vormen een aanzienlijke bron van boven- en ondergrondse biodiversiteit, hebben landschappelijke meerwaarde en spelen een belangrijke rol in **waterregulatie**. Klimaatverandering doet neerslagpatronen verschuiven, daardoor neemt de kans op zowel piekregens als perioden van droogte toe, met als gevolg onder meer oogstverliezen, wateroverlast en schade aan infrastructuur. De frequentie en intensiteit van deze weersextremen neemt naar verwachting toe in de toekomst. Dit betekent dat het waterregulerend vermogen van de bodem, gedefinieerd als de **capaciteit van de bodem om water op te nemen, vast te houden en te leveren**, van toenemend belang is.

Het waterregulerend vermogen van de bodem wordt met name bepaald door bodemeigenschappen. Sommige van deze eigenschappen zijn nagenoeg onveranderlijk, zoals de textuur en mineralogie; andere eigenschappen, zoals structuur en organisch stofgehalte kunnen worden beïnvloed door o.a. plant-bodeminteracties, bodemleven en mechanische grondbewerking. Sommige bodems zijn gevoeliger voor piekregens en droogte dan andere. In Nederland zijn bijvoorbeeld de hoge zandgronden in het zuiden en oosten van het land kwetsbaar voor beide. De ± 4.500 melkveehouders alsmede de waterschappen in deze gebieden zijn op zoek naar manieren om het waterregulerend vermogen van hun bodems te verbeteren. Bodembeheer en bodemleven, met name **regenwormen**, kunnen een belangrijke bijdrage leveren.

Onderzoek heeft laten zien dat pendelende regenwormen zoals *Lumbricus terrestris* kunnen bijdragen aan een verbeterde waterinfiltratiesnelheid en -capaciteit van de bodem, dankzij de diepe verticale gangen die ze graven. Deze soort doet het goed in grasland, is inheems voor Europa en komt voor op alle bodemtypen die Nederland rijk is. Desalniettemin wordt *L. terrestris* op het niveau van het perceel niet overal gevonden of komt de soort soms voor in hele lage populatiedichtheden. Er is veel potentie voor verbeterde waterregulatie op grasland door het stimuleren of introduceren van *L. terrestris*-populaties. Het doel van het onderzoek in dit proefschrift was om meer inzicht te krijgen in de **habitatgeschiktheid** van grasland op zand voor *L. terrestris* en ook om de mogelijkheden van **beheersmaatregelen** en **regenwormenintroductie** te verkennen met als uiteindelijk doel bij te dragen aan de klimaatadaptatie van grasland.

In **Hoofdstuk 2** richtten we ons op de vraag welke eigenschappen van grasland op zand van invloed zijn op de aanwezigheid en abundantie van *L. terrestris*. Een beter begrip van de eisen die de soort stelt aan zijn omgeving zouden namelijk helpen te bepalen welke graslanden geschikt voor ze zijn en welke beheersmaatregelen genomen zouden kunnen worden om de populatie te stimuleren. Data werd verzameld middels een veldinventarisatie van *L. terrestris* in graslanden van melkveehouders op zandgrond in het zuiden van Nederland. Vervolgens is gezocht naar correlaties tussen de aanwezigheid en de abundantie van *L. terrestris* met bodemtextuur, grondwatertrap, interspecifieke competitie met strooiselbewonende regenworm *Lumbricus rubellus* en het aantal verstreken jaren sinds de laatste mechanische bodemverstoring (leeftijd van het grasland). *L. terrestris* werd gevonden in graslanden met uiteenlopende bodemtextuur, grondwatertrappen, soortsamenstellingen en leeftijden. Abundantie liet een positieve correlatie zien met het aandeel leem in de bodem op 30-40 cm diepte en een negatieve correlatie met de abundantie van *L. rubellus*.

In **Hoofdstuk 3** onderzochten we het effect van graslandbeheer op de populatiedynamiek van regenwormen, specifiek het effect van graslandvernieuwing. Graslandvernieuwing is een belangrijke maar weinig onderzochte beheersmaatregel. Deze wordt met name ingezet om de productiviteit en botanische compositie van grasland te verhogen. De procedure bestaat uit het vernietigen van de bestaande zode door middel van herbiciden of ploegen, gevolgd door herinzaai. Herinzaai vindt ofwel direct plaats, of na één of meerdere seizoenen van akkerbouw – vaak voedermaïs – in plaats van gras. Data werd verzameld in twee opeenvolgende jaren in een veldexperiment op een melkveehouderij waar mesocosms geplaatst waren. Het effect van verschillende graslandvernieuwingsstrategieën op de abundantie, demografie en soortsamenstelling van de regenwormenpopulatie werd getest. De strategieën omvatten een combinatie van behandelingen met en zonder ploegen en met en zonder verlies van de vegetatielaag. Na zes maanden werd een duidelijk effect gevonden op abundantie, deze nam af met een toenemende mate van verstoring (van lage naar hoge verstoring: permanent grasland, geen herinzaai > niet ploegen, vegetatielaag verwijderd zonder herinzaai > wel ploegen, met herinzaai van gras > wel ploegen, geen herinzaai). Ploegen had een sterker negatief effect dan het verlies van de vegetatielaag op regenwormabundantie, hun gecombineerde effect was het meest schadelijk. Met name het aantal bodembewoners (*Aporrectodea caliginosa* en *Aporrectodea rosea*) nam af onder invloed van ploegen en het verlies van de vegetatielaag. Strooiselbewonende en pendelende regenwormen waren

überhaupt minder abundant en deze groepen werden minder duidelijk beïnvloed door de behandelingen. Ploegen en verlies van de vegetatielaag liet na zes maanden een groter effect zien op het aantal adulte dan juveniele regenwormen, er was geen invloed van de behandelingen op de soortendiversiteit.

In de Hoofdstukken 4 en 5 lag de focus op de introductie van *L. terrestris* in grasland op zandgrond. **Hoofdstuk 4** beschrijft een veldexperiment waarin *L. terrestris* is geïntroduceerd werd in mesocosms op twee melkveehouderijen. In geen van beide graslanden was voorafgaand aan de introductie een *L. terrestris*-populatie aanwezig, maar de percelen leken daar wel geschikt voor op basis van inzichten uit de literatuur en de inventarisatie uit Hoofdstuk 2. De totale duur van het experiment was 15 maanden, en een deel van de geïntroduceerde *L. terrestris* bleek in staat om in deze periode te overleven, nakomelingen te produceren en gangen van > 60 cm diep te graven. Het overlevingspercentage was 32% na zeven maanden en 6% na 15 maanden. We vonden indicaties van interspecifieke competitie met *L. rubellus*, ten nadele van *L. terrestris*. Verschillende mogelijke redenen voor de hoge sterfte onder de geïntroduceerde regenwormen werden voorgesteld: droogte, aangezien de zomer erg droog was; competitie om voedsel met *L. rubellus*; en een beperkt aanpassingsvermogen van de commercieel verkregen *L. terrestris* (geïmporteerd uit Canada).

Een vervolgexperiment werd uitgevoerd in **Hoofdstuk 5**, hierin testten we hypotheses die voortkwamen uit Hoofdstukken 2 en 4. Een vergelijkbare mesocosm-opzet werd gebruikt, maar met enkele aanpassingen. Ten eerste werd de voedselbeschikbaarheid verhoogd om competitie met *L. rubellus* te voorkomen. Ten tweede werd er gebruik gemaakt van *L. terrestris* met verschillende herkomst, namelijk commercieel verkregen (uit Canada) en lokaal verzamelde individuen. Ten derde, *L. terrestris* werd geïntroduceerd in een grasland waar de soort tot dusver absent was én in een grasland dat al een eigen populatie had. Introductie in een grasland met bestaande populatie diende ertoe onderscheid te kunnen maken in het effect van de geschiktheid van de habitat en de effecten van de introductieprocedure. Dankzij het gebruik van VIE tags waren de geïntroduceerde regenwormen te onderscheiden van de residentiële populatie. Het experiment duurde 12 maanden. Net als in het experiment van Hoofdstuk 4 bleek een deel van de regenwormen in staat te zijn geweest om te overleven, nageslacht te produceren en gangen van > 60 cm diep te graven. Het overlevingspercentage was 15% in het grasland mét eigen populatie en 26% op het grasland zonder eigen populatie. De lokaal verzamelde *L. terrestris* presteerde iets beter dan de Canadese exemplaren.

De vergaarde inzichten uit Hoofdstukken 2-5 werden geïntegreerd met elkaar en de bestaande literatuur in **Hoofdstuk 6**. Daarnaast worden in dit hoofdstuk enkele aanvullende bevindingen uit het onderzoek besproken, namelijk: de voorwaarden waaronder zandgrond een geschikte habitat voor *L. terrestris* kan zijn; hoe regenwormenpopulaties in sommige van de experimenten verrassend goed herstelden van ernstige verstoringen; tot slot, hoe historisch landgebruik en beperkte toegankelijkheid van graslanden zou kunnen leiden tot absentie van *L. terrestris* op plekken die in principe wel geschikt zouden zijn voor de soort. Vervolgens is de invloed van de onderzoeksmethode op de resultaten bediscussieerd en werden enkele suggesties voor toekomstig onderzoek gegeven. Ten eerste, non-destructieve methodes voor het bemonsteren van regenwormen zouden monitoring kunnen verbeteren. Ten tweede, meer data met betrekking tot bodemvocht zou nuttig zijn. Ten derde, mesocosms blijken erg geschikt voor het type onderzoek in dit proefschrift, als haalbaarheidsstudie, de volgende stap zou zijn om langetermijnexperimenten in een openveldopzet uit te voeren.

In de afsluitende sectie plaatste ik dit onderzoek in een breder perspectief met nadruk op het belang van een systemische benadering bij vraagstukken rondom klimaatadaptatie. Praktische implicaties voor onderzoekers, boeren, regenwormenkwekers en waterschappen werden besproken. Ik concludeerde dat er potentie is voor het actief inzetten van *L. terrestris* ter bevordering van het waterregulerend vermogen van graslanden op zandgrond. Wetenschappelijke expertise, pragmatische kaders, praktijkervaring, een netwerk en financiële middelen zullen moeten worden samengebracht om de praktische haalbaarheid en succesvolle strategieën voor beheersmaatregelen en regenwormenintroducties te onderzoeken in de praktijk. Zo kan een volgende stap gezet worden in het bevorderen van de klimaatadaptatie van grasland.



Chapter 1

General introduction

1. General introduction

1.1 Background: grasslands for climate adaptation

Agricultural grasslands deliver more ecosystem services than is generally acknowledged. Grasslands not only provide feed for animals, but they also play an important role in climate change mitigation through carbon storage (Bai & Cotrufo, 2022; Jones & Donnelly, 2004), their contribution to above and belowground biodiversity can far exceed that of arable land (Van Eekeren, De Wit et al., 2025; Werling et al., 2014), they are part of cultural heritage (Eriksson, 2022; Pellaton et al., 2022), and they contribute to **water regulation** (Sirimarco et al., 2018; Van Eekeren et al., 2025a). Water regulation, defined as the **capacity to absorb, retain and supply water**, is becoming increasingly important. As precipitation patterns are shifting, the incidence of peak rains and prolonged droughts is rising (Santos et al., 2016), resulting in harvest losses, floods and infrastructural damage. The prevalence and intensity of such severe weather events is estimated to increase due to climate change (Grillakis, 2019; Samaniego et al., 2018; Tradowsky et al., 2023). Therefore, it is of the utmost importance that the water-regulating capacity of grasslands is improved and maintained. Grasslands cover about 25% of the earth's total land surface and 70% of all agricultural land worldwide, and thus have an enormous potential. In the EU alone, grasslands cover 48 million ha, which is 34% of the EU's agricultural land (Eurostat, 2018).

This thesis focuses on intensively managed **temperate grasslands on sandy soil**. There is a strong incentive to find climate adaptive strategies for these grasslands as they offer a range of ecosystem services for society (Figure 1.1) and they are economically important, as a result of their central role in dairy farming systems. These grasslands are threatened by peak rains and droughts in various ways. When the amount of precipitation exceeds the water infiltration capacity of the soil, waterlogging and surface runoff may occur. Waterlogging compromises plant productivity and increases N_2O emissions (Reyer et al., 2013; Sairam et al., 2008). Runoff leads to nutrient losses, which is detrimental to surface water quality. In severe cases, runoff may cause floods, leading to huge damage to infrastructure, nature and harvest yields. Europe has suffered from a series of severe and sometimes deadly floods in the past decade, for example, in The Netherlands (2016 and 2021), Belgium (2021), Germany (2021), Central Europe (2024), Switzerland (2024) and Spain (2024 and 2025). Simultaneously, drought has had negative effects on European crop and grass production in recent years (Beillouin et al., 2020; Fu et al., 2020; Van Oort et al., 2023). In addition,

competition for water between stakeholders is increasing, as nature, water boards and drinking water companies are all experiencing the consequences of drought. Thus, **climate adaptive soil measures are necessary** to ensure safe and sustainable water regulation under future climatic conditions.

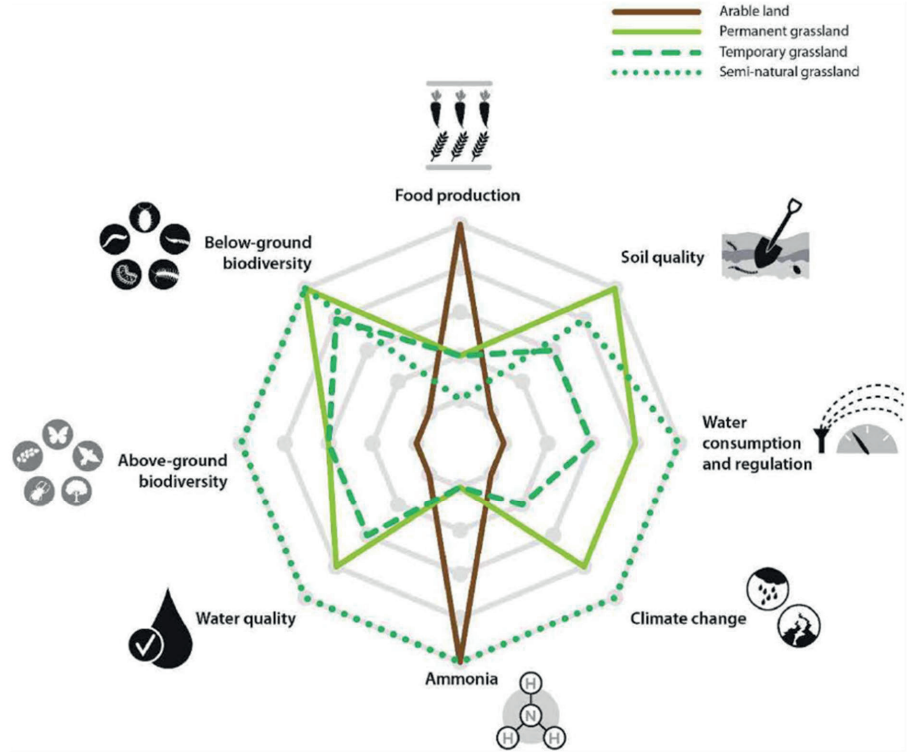

Figure 1.1 The score of different land-use types on ecosystem indicators on a scale of 1-5. The most positive land-use type scores 5, the others are scored relative to this (From Van Eekeren, De Wit et al., 2025).

The water-regulating capacity of grassland is mainly determined by its soil properties. Some of these properties are virtually unchanging, such as texture and mineralogy. Other properties are influenced by soil management, plant-soil interactions, soil biota and natural processes (Jenny, 1994; Veenstra & Lee Burras, 2015). Inconstant properties that have an effect on water-regulating capacity include structure, organic matter content, aggregate formation, aggregate stability and soil porosity. Such inconstant properties enable active improvement of the water-regulating capacity (Warrick, 2001). Soil management practices can have both direct and indirect effects on soil properties. For example, tillage not only affects soil structure directly through mechanical soil manipulation, but also indirectly, as it affects the soil biota that in

turn have an effect on the soil structure. In the following sections, I zoom in on the effect of earthworms on soil quality, with a special emphasis on **deep-burrowing earthworms** and their effect on water regulation. I then discuss how grassland-related management practices can affect earthworm communities, and lastly I present the aims of this thesis.

1.2 Soil biota and grassland ecosystem functioning

Soil biota are drivers of many soil processes and consequently play a crucial role in soil functioning (Morris & Blackwood, 2024). The relationships between different organisms give rise to the soil food web, and many of the complex interactions between its members result in what from an anthropocentric viewpoint are considered ecosystem services. This is also the case in grasslands, where an intimate link exists between the sward and its associated biotic community, the **plant-soil interactions** that arise from it work both ways. On the one hand, grasses feed soil organisms through root exudates and litter from roots and leaves. In a perennial ryegrass sward, 4,500 kg organic root mass ha^{-1} is decomposed on a yearly basis (Deinum, 1985). It was calculated that approximately 30-70% of the net fixed C in perennial grasses is transferred below ground to feed the biotic community (Lynch & Whipps, 1990). On the other hand, grasses benefit from soil organisms as they improve nutrient availability (Brussaard et al., 2007), suppress pests (Schlatter et al., 2017) and provide soil structure for the plant roots (Oades, 1993). Nevertheless, the importance of soil biota, such as earthworms, has often been overlooked (Van Eekeren et al., 2007). This research aims to improve our understanding of earthworm populations in grasslands on sandy soil.

1.3 Earthworms and their ecosystem services

In temperate grasslands, earthworm population densities can reach > 1,000 individuals m^{-2} and on Dutch dairy farms, the earthworm biomass ha^{-1} of a grassland sometimes exceeds the biomass ha^{-1} of the cows that graze it (Van Eekeren et al., 2007). It has long since been established that earthworms play a key role in grassland ecosystem functioning. As early as 1789, at a time when earthworms were regarded as garden pests by the general public (Butt, 2023) and long before the term *ecosystem services* existed, the naturalist Gilbert White was the first to acknowledge that earthworms are crucial for soil fertility and food production (Holt-White, 1901). White would become a source of inspiration for Darwin, who greatly improved the appreciation for earthworms when he published his seminal work "*The formation*

of vegetable mould through the action of worms with observations on their habits" in 1881. However, it took another century before earthworm research really began to take off.

In 1994, the term **ecosystem engineers** was coined (Jones et al., 1994), and earthworms have been considered a textbook example ever since. Ecosystem engineering is defined as *the direct or indirect modulation of the availability of resources (other than the species itself) to other species, by causing physical state changes in biotic or abiotic materials; by doing so, they modify, maintain and/or create habitats*. Earthworms engineer ecosystems by burrowing, feeding and casting and since they were recognised for doing so in 1994, scientific output concerning earthworms and ecosystem services has been steadily increasing. Numerous studies have delved into the intricate interactions between earthworms and their habitat in agricultural settings (Blouin et al., 2013; Chan, 2001; Geisen et al., 2019; Giller et al., 1997; Lori et al., 2025).

While burrowing through the soil, earthworms create macropores, promoting aeration and connectivity. It is well known that earthworms modify the **hydraulic properties** of the soil (Blouin et al., 2013). Although it is difficult to quantify the effect of earthworms on soil hydraulic properties, a number of studies have shown that earthworm activity promotes the soil's water infiltration rate (Bouché & Al-Addan, 1997; Capowiez, Cadoux, Bouchant et al., 2009; Deru et al., 2018; Ehlers, 1975; Guéi et al., 2012) and water-holding capacity (Hallam & Hodson, 2020). In grasslands on peat, Deru et al. (2018) showed a significant relationship between earthworm abundance, the number of macropores and water infiltration (Figure 1.2). Earthworms also promote aggregate formation and stabilisation (Angst et al., 2019; Six et al., 2004). They do so by stimulating microorganism activity, which leads to an increase in the microbial glue (i.e., microbial cell walls and extracellular polymeric substances) with which mineral particles and microaggregates cohere into macroaggregates. Moreover, earthworm activity creates hotspots that are enriched in microbial cell walls, facilitating the formation of new microaggregates (Angst et al., 2019). Aggregation has a positive impact on soil structure, as stable aggregates make the soil less susceptible to sealing (Lado et al., 2004), compaction (Baumgartl & Horn, 1991) and nutrient leaching (Blanco-Canqui & Lal, 2004).

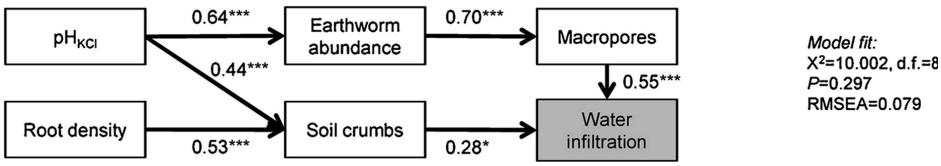

Figure 1.2 Earthworm abundance significantly increases the number of macropores, which significantly contributes to water infiltration in grasslands on peat (From Deru et al., 2018).

Apart from water regulation, the feeding and casting behaviour of earthworms is of vital importance for soil formation, nutrient cycling and plant primary production (Bhadauria & Saxena, 2010; Blouin et al., 2013). Soil formation is a slow process, subject to the interplay of time, topology, climatic conditions and the nature of the parent material (Chesworth, 1992); however, biotic activity also plays a role (Finlay et al., 2020). As earthworms feed, burrow and cast, they mix and redistribute organic and mineral matter (Darwin, 1881; Frouz, 2024). Earthworms feed on organic matter such as plant litter, dung, root exudates, soil organic matter, fungi and bacteria. As they ingest, comminute, digest and excrete, they accelerate the degradation of organic matter and molecules (Blouin et al., 2013). Some of the metabolites produced are released into the environment as small organic compounds or nutrients in mucus, dead tissue and worm-casts, and these then become available to plants (Chaoui et al., 2003; Vidal et al., 2019). Worm-casts are generally more fertile than bulk soil, as they are rich in nitrogen, phosphorous and have increased Cation Exchange Capacity (CEC) (Van Groenigen et al., 2019; Vos et al., 2019). Nutrients are not only made available, but they are also transported horizontally and vertically through the soil while earthworms move around and commute between the soil surface and deeper layers. Additionally, as plant litter and dung is consumed by earthworms, it is removed from the soil surface, which prevents the suffocation of vegetation under a dung patch or litter mat.

Earthworm-mediated processes contribute significantly to **plant primary production**. A meta-analysis by Van Groeningen et al. (2014) demonstrated that aboveground biomass of pasture grasses was significantly higher in the presence of earthworms (+24%). Schon et al. (2020) set out to calculate the monetary contributions of earthworm-mediated ecosystem services to a New-Zealand pastoral system grazed by dairy cattle. They calculated the added value $year^{-1}$ ha^{-1} in a scenario with either low abundance and diversity or high abundance and diversity of earthworms. Among the ecosystem services assessed were provision of food quantity (612-1265 NZD),

flood mitigation (164-493 NZD) and carbon storage (6-78 NZD), leading to a total of 900-2338 NZD $year^{-1}$ ha^{-1}.

Earthworms deliver many beneficial ecosystem services, but also **trade-offs** exist. Improved water infiltration as a result of earthworm burrowing can have the negative side-effect of increased nutrient leaching, as was demonstrated in a field study on arable land (Domínguez et al., 2004). A lab and mesocosm study revealed that elevated CO_2 and N_2O emissions in the presence of deep-burrowing earthworms increased the global warming potential (GWP) of an arable field. This was explained to be a consequence of higher macrofaunal densities in the vicinity of burrows, combined with their elevated content of mineral nitrogen and soil moisture (Nieminen et al., 2015). A field study in temperate grassland showed that the presence of the epigeic earthworm *Lumbricus rubellus* strongly increased N_2O emissions in autumn, but not in spring (Lubbers et al., 2013). However, in a later study, Lubbers et al. (2019) found soil N_2O emissions to reduce with increased species richness and functional dissimilarity of soil organisms, including earthworms. This was attributed to more complete denitrification due to the presence of more diverse soil biota. A better understanding of these complex and context-dependent processes is necessary to weigh the advantages and disadvantages of altering earthworm populations on a large scale.

1.4 Not all earthworms are equal

One of the important lessons learned from previous research is the need to acknowledge the **diversity in functional traits** between earthworms species (De Lange et al., 2013; Pelosi et al., 2014). A recent estimate by Decaëns et al. (2025) suggests that the total number of earthworm species worldwide could be as high as 30,000, of which 5,679 taxa have currently been described. Worldwide, there are great differences between species. Adult sizes range from less than a millimetre (genus *Chaetogaster* von Baer, 1827) to over 2 metres (*Megascolides australis*). Preferred habitats range from wood (*Bimastos rubidus*) to wetland (*Sparganophilus eiseni*). Reproductive cycles range from weeks (*L. rubellus*) to months or even more than a year (*Lumbricus terrestris*). Within a given habitat type, earthworms species assemblies mostly consist of only a small number of species. In European forests, species diversity was found to fall within a range of 4 to 15 (Cesarz et al., 2007; De Wandeler et al., 2018; Nachtergale et al., 2002), 4 to 6 species were found in tropical grasslands and forests (Decaëns et al., 2004, 2009; Phillips et al., 2019) and 5 to 15 species in temperate grassland (Curry et al., 2008; Hoeffner, Santonja et al., 2021; Hoeffner et al., 2024; Piotrowska et al., 2013).

Within a specific species assembly in a given habitat type, in this case temperate grassland on sandy soil, species with different functional traits occur. For example, species with large and small body sizes may co-occur and deploy contrasting survival strategies. On an individual scale, a large body size allows for deeper burrowing to avoid adverse conditions near the surface, whereas at a population scale, a small body size allows for quick maturation and reproduction to recover from adverse conditions. This variation results in the stable co-existence of several species in a single habitat, indicating that these species occupy distinct niches, which requires specialisation. As a consequence, different earthworm species make their own unique contributions to engineering an ecosystem. Therefore, it is crucial to not only consider earthworm abundance, but to also take **functional diversity** into account when researching the potential for earthworm communities to support ecosystem services.

In the early 1970s, Bouché proposed three ecological groups of earthworms, **epigeics, endogeics and anecics** (Bouché, 1971). This classification was based on differences in (1) vertical distribution, (2) ecology and (3) morphology, and it soon became a standard in earthworm research (Bottinelli et al., 2020). In this thesis, I use this classification to distinguish between three broad ecological categories, based on literature and personal observations in the field. The following characteristics are attributed to these categories (Figure 1.3):

- *Epigeics* are pigmented and relatively small earthworms that feed mostly on aboveground plant litter. Generally, they can be found in the first 20 cm of the soil.
- *Endogeics* are greyish earthworms, small to medium in size. They feed mostly on soil organic material and most of them are found in the first 40 cm of the soil, although they sometimes move deeper.
- *Anecics* (or deep-burrowing earthworms) are partly pigmented earthworms that can be medium to large in size. They are able to dig deep burrows and can sometimes be found at over 2 metres deep, depending on species and soil conditions.

In recent years, a number of researchers in the field have expressed their concerns about the casual use of this terminology (Bottinelli et al., 2020; Bottinelli & Capowiez, 2021; Capowiez et al., 2024). One important point is that these three groups represent ecological categories rather than functional groups (Bottinelli et al., 2020). In this thesis, we have decided to use the terms epigeic, endogeic and anecic in their more

'classical' sense, which might not be completely up-to-date with emerging insights about ecological categories (Bottinelli et al., 2020) and functional groups (Capowiez et al., 2024). However, I consider the risk of over-generalisation limited as the studies presented in this thesis concern a modest number of earthworm species and only a single habitat type is covered.

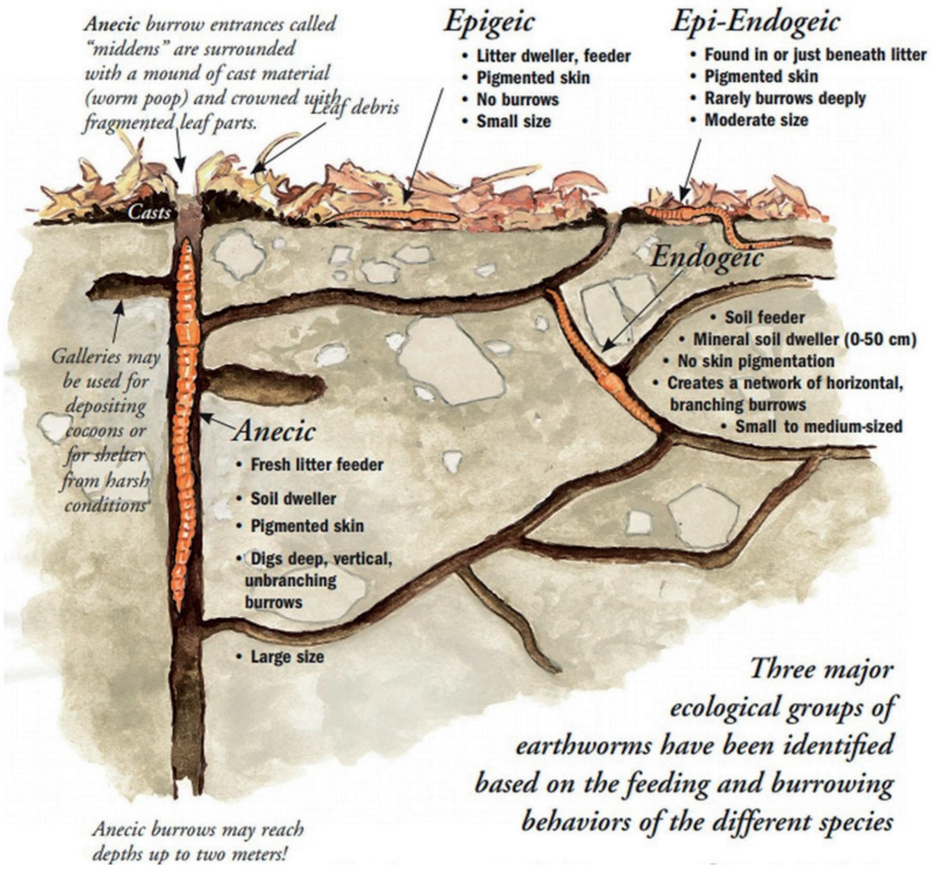

Figure 1.3 Illustration by Rick Kollath, retrieved from https://www.earthwormsoc.org.uk/earthworm-ecology. A simplified overview of earthworm ecological groups, their feeding preferences, and burrowing behaviour.

1.5 *Lumbricus terrestris*: more equal than others

L. terrestris is a relatively well-studied representative of anecic earthworms. In the Netherlands, it is the most abundant deep-burrowing species (Rutgers et al., 2016). It plays an important role in grassland ecosystem functioning and has a large potential for supporting climate adaptation. Previous research has shown that their **deep**

and vertical burrows improve the water infiltration rate as well as the infiltration capacity of the soil. An increase of 100 mm year^{-1} added infiltration was measured in the field when *L. terrestris* was abundant (W. M. Edwards et al., 1990). The introduction of *L. terrestris* in no-till fields was followed by a significant increase in water infiltration (Willoughby & Kladivko, 2002). Compared to four endogeic species, the infiltration rate in soil cores with *L. terrestris* was 2-5 times higher (Capowiez et al., 2015). **Water-holding capacity** in mesocosms filled with sandy loam increased by 11% in the presence of *L. terrestris* (Hallam & Hodson, 2020). Therefore, introducing *L. terrestris* can assist in flood and runoff mitigation while replenishing groundwater. Additionally, *L. terrestris* burrows facilitate **deeper plant rooting** (C. A. Edwards & Lofty, 1978; Pitkänen & Nuutinen, 1997), which may improve drought resistance because of better access to deeper soil moisture.

There is a clear need for an improvement in the water-regulating capacity of agricultural grasslands on sandy soil. *L. terrestris* could offer a nature-based solution to help tackle this challenge. However, there are current knowledge gaps regarding the presence and abundance of *L. terrestris* in relation to soil parameters. Within the context of temperate grassland on sandy soil, this raises the question: *which requirements need to be met for* L. terrestris *to thrive*? This thesis aims to improve our understanding of what makes grassland suitable for *L. terrestris*, in terms of texture, management, hydrology and interspecific competition. This information could then be used in taking soil management decisions to predict or stimulate the occurrence of *L. terrestris* in grasslands, or even to inoculate grasslands with *L. terrestris*, with the ultimate goal of supporting climate adaptation. First, however, I evaluate our current knowledge of *L. terrestris*.

1.5.1 Ecology of *L. terrestris*

L. terrestris is considered a species **native to Western Europe**. Although it disappeared from the northern part of the continent during the last glacial maximum (25,000-10,000 years ago), it has since then recolonised the area (Tiunov et al., 2006). *L. terrestris* has a unique ecology that is distinct from the rest of the earthworm species that occur in Dutch agricultural grasslands. Whereas all other earthworms in these grasslands are usually found in the first 40 cm, *L. terrestris* can go deeper, in some cases as far as 2 m into the soil (Edwards and Bohlen, 1996). A burrow is generally vertical (Jégou et al., 1999; Shipitalo & Gibbs, 2000) and inhabited by a single individual. The inside of the burrow is lined with a mucus rich layer (Andriuzzi et al.,

2013) to make commuting easier while simultaneously protecting the earthworm from abrasive soil particles and desiccation. These earthworms allocate much time and energy to the construction of a deep burrow because burrows are important for their survival. A burrow provides access to deep soil layers, where moisture and temperature conditions are much more stable than just below the surface (although high groundwater levels can be problematic when burrows become inundated and deprived of oxygen). Deep burrows also offer protection against predators. It is one of the largest earthworm species found in Europe, and the single largest species in the Netherlands. With an average adult weight between 2 and 7 gr, *L. terrestris* makes an ideal prey for moles, birds, badgers and other animals (Macdonald, 1983). The size and weight they reach at maturity is partly determined by food quality and availability (Butt, 2011b).

L. terrestris is more commonly known as the *nightcrawler* or *dew worm*, and this refers to its predominantly **nocturnal activity**. Whereas the day is spent mostly within the safety of the burrow, the period between dusk and dawn is used for collecting food from the soil surface and finding a partner for mating. During both activities, *L. terrestris* prefers to keep its tail in its burrow. The flattened end of its tail enables *L. terrestris* to anchor itself to its burrow, ready for a rapid retreat in case of danger. Part of the food that is collected is left gathered around the entrance of the burrow, forming a **midden**. The midden seems to be a multifunctional structure; it protects the cavity of the burrow from water evaporation and as it is made of organic material, it forms a hotspot for microbial activity. The latter is important for *L. terrestris*, as microbial activity helps to pre-digest its food. In agricultural grasslands, these earthworms feed mostly on plant litter (Griffith et al., 2013; Valckx et al., 2011) and manure or other organic inputs.

The **life cycle** of *L. terrestris* is long compared to other species within this habitat type as *L. terrestris* is more K-selected than other *Lumbricidae* (Daniel, 1992). Its life cycle is not easy to study under field conditions; as a consequence, most of the available literature about the *L. terrestris* life cycle and the time it takes for the species to go through various developmental stages was gained in laboratory conditions. It was found that when two adults mate successfully, they can both produce cocoons, which can be done immediately or after a period of time. *L. terrestris* is able to conserve received sperm and produce viable cocoons for over one year after mating (Butt, 2011b). The cocoon production rate can be over 4 cocoons month^{-1} in the lab but is expected to be lower in the field. Hatching time in the lab is extremely variable,

showing a range of 3 to 23 months, depending various conditions, including temperature and moisture (Butt, 2011b, 2011c). After hatching from its cocoon, *L. terrestris* takes a minimum of 12 weeks to reach sexual maturity under ideal conditions (Butt et al., 1992; Butt, 2011b). In the field, maturation has been shown to take longer, sometimes as long as approximately one year (Daniel, 1992). In culture, *L. terrestris* has been found to live up to eight years, under natural conditions, its longevity is almost certainly less (Lakhani & Satchell, 1970).

1.5.2 Heterogeneous distribution and slow dispersal of *L. terrestris*

L. terrestris can be found in all three main soil types of the Netherlands, namely sand, clay and peat (Rutgers et al., 2016; Van Eekeren et al., 2014). In temperate grasslands, the species can thrive and reach population densities of up to several hundreds of individuals m^{-2} (including juveniles) (Daniel, 1992; Rutgers et al., 2016). It is likely that they reach their highest abundances in grassland because of the steady food supply, low level of soil disturbance and the protective vegetation layer. However, the presence and abundance of *L. terrestris* is heterogeneous and difficult to predict at the field and landscape scale. Differences in habitat suitability are an obvious driver for variation, but also slow or discontinuous dispersal could lead to a heterogeneous distribution. The contribution of all three are explored below.

Earlier attempts to explain and predict *L. terrestris* **distribution** have resulted in a set of parameters that aims to explain *L. terrestris* occurrence (Lindahl et al., 2009; Pelosi et al., 2008), but not specifically for grassland on sandy soil, which is a narrow but important ecological context. WORMDYN is a model for *L. terrestris* population dynamics that was created by Pelosi et al. (2008), and which focuses on arable land. Lindahl et al. (2009) included grassland when they gathered the outcomes of 39 studies on 86 agricultural sites across Europe. They created an overview of *L. terrestris* presence and abundance in relation to various factors, including climate, depth restrictions, hydrologic conditions, land use, texture, tillage system, organic additions and pH. The most determining factors were land use and texture, and the authors created a two-step classification tree to predict *L. terrestris* abundance (step 1 > Land use: perennial or ley/arable; step 2 > Texture: fine, medium or coarse). We aim to use their research as our starting point and increase the resolution of our knowledge by zooming in on perennial grasslands on sandy soil.

The **natural dispersal** of *L. terrestris* is slow. Firstly, these earthworms spend most of their time in the safety of their burrow. When they leave it, this is generally with

the purpose of finding a partner to mate with. However, as mentioned above, even during mating their tail is often anchored in a burrow (C. A. Edwards & Bohlen, 1996; Nuutinen & Butt, 1997). In a lab study, *L. terrestris* seldom left its burrow completely; this happened in only 4% of all surface visits. In the cases that it did leave its burrow completely, surface activity was commonly ended by settling in a pre-existing burrow (78% of the cases) and sometimes by returning to its original burrow by moving backwards along its trail (16%) (Mather & Christensen, 1988; Nuutinen & Butt, 2005). Consequently, only 6% of these surface visits ended in the creation of a new burrow. The behaviour observed in this lab study suggests a low dispersion rate of *L. terrestris*, as neither settling in a pre-existing burrow or homing contribute to dispersal. Additionally, the relatively lengthy reproduction cycle and maturation time of *L. terrestris* (Butt, 2011b; Daniel, 1992) are likely to slow down natural dispersal.

Field studies reported **dispersion rates** of 1.1 m $year^{-1}$ in arable heavy clay under boreal conditions (Nuutinen et al., 2006), of 1.4 m $year^{-1}$ in temperate Dutch grassland (Ligthart & Peek, 1997) and 4.5 m $year^{-1}$ (Hoogerkamp et al., 1983) in temperate Dutch grassland on a silt loam soil, and of 6 m $year^{-1}$ in an arable system on silt loam under a humid continental climate (Shuster et al., 2003). However, the field observations of Mather and Christensen (1988) paint a slightly different picture, as they found surface movements of *L. terrestris* to cover a mean distance of 9 m in one night. Unfortunately, their paper does not clarify whether the surface visits of these earthworms ended in pre-existing burrows – thus remaining within an already colonised area – or whether the earthworms created new burrows at the end point of their journey, thus possibly entering previously uncolonised terrain. The maximum distance covered by a single individual in one night was 19.3 m. Although these distances are considerable, it should be mentioned that the tracks were mostly curved, so even though the total length of the trail was 9 m on average, the distance between the starting and the finishing point was smaller, but not specified by the authors. Earthworms tend to keep close to their starting point (Marinissen & Van den Bosch, 1992).

Several factors could contribute to **discontinuous dispersal**. First, **fragmentation** of the landscape may play a role. If current and potential habitats are separated by roads and waterways, it may be impossible for *L. terrestris* to move from one to the next habitat independently. In combination with its slow dispersal rate, habitat fragmentation may simply prevent the species from reaching new territory. Second, the haphazard nature of **anthropogenic dispersal** may be a contributing factor; this is sometimes associated with purposeful inoculation (Butt, 2011c; Petit-dit-Grézériat

et al., 2024a; Stockdill, 1966a) but mostly happens unintentionally. Earthworms and cocoons may be transported to uncolonised terrain in soil attached to planting material, tyres of cars and agricultural machinery, and discarded fishing bait (Baumann et al., 2024). When earthworms and/or cocoons are introduced to a new field, they need to adapt to their new environment, avoid predators, avoid desiccation, find enough food and adjust to new microbial interactions, and all of these pose quite a challenge. Unintentionally inoculated earthworms will only survive if the circumstances allow so, the earthworms need to be strong and adaptive enough to cope with a sudden change in environment. Whereas natural dispersal is more continuous and gradual in nature, anthropogenic dispersal may occur more haphazardly and therefore be discontinuous in space and time.

Overall, I hypothesise that the absence of *L. terrestris* from a given grassland on sandy soil does not necessarily indicate that the grassland is unsuitable for the species. Given the slow natural dispersal rate of *L. terrestris* and discontinuous anthropogenic dispersal, it seems plausible that some grasslands are out of reach for existing and expanding populations. Inoculation could be a strategy to initiate or speed up the establishment of an *L. terrestris* population. However, we have insufficient knowledge of the soil characteristics that determine whether a given grassland on sandy soil is suitable to host a population of *L. terrestris*. Once we know the requirements of the species, we will be able to assess whether a grassland on sandy soil could host an *L. terrestris* population. Understanding the species' habitat requirements might also help us understand which management practices and inoculation methods could be effective in promoting the presence and abundance of *L. terrestris*.

1.5.3 Agricultural management practices and their effects on earthworms

Earthworms and agriculture have an ambivalent relationship. On the one hand, when farmers maintain permanent grassland and add organic fertilisers, they provide a much richer environment than a natural system would. On the other hand, when farmers apply regular tillage and pesticides, they might cause the demise of complete earthworm populations. In effect, it is well-known that agricultural management practices affect soil biota and the functions that they support (Lori et al., 2025). Much effort has been dedicated to unravelling the complex relationships between agricultural soil management practices and their effect on soil biota such as earthworms, but many unanswered questions remain due to the scope and complexity of the topic.

It is beyond the span of this thesis to give a full overview of the current knowledge concerning the effects of agricultural management practices on earthworm populations. Recent reviews and meta-analyses have taken on this challenge (Edwards & Arancon, 2022; Lori et al., 2025). Here, I will narrow it down to a number of practices that are related to intensively managed grassland and their effects on earthworm populations. Earthworm research has been conducted on grassland age (i.e. years without soil tillage) (Hoeffner, Santonja et al., 2021; Krogh et al., 2021), stocking rate (Cluzeau et al., 1992; Hoeffner, Santonja et al., 2021; Hoeffner et al., 2024; Schlaghamerský et al., 2007; Schon et al., 2021), tillage combined with conversion to arable (Edwards & Lofty, 1982; Low, 1972; Postma-Blaauw et al., 2012), botanical composition (Baekelmans et al., 2026; Eisenhauer et al., 2009; Fischer et al., 2014) and types and levels of fertilisation (Bacher et al., 2018; Cozim-Melges et al., 2024; Curry et al., 2008; Deru et al., 2023; Hoeffner, Santonja et al., 2021; Timmerman et al., 2006; Uvarov, 2009; Van Eekeren et al., 2009a). Below I briefly address these factors; subsequently, I discuss the specific agricultural management practice of grassland renewal and its effects on earthworm populations.

Grassland age has been found to correlate positively with earthworm abundance and biomass (Krogh et al., 2021; Van Eekeren et al., 2008), which is probably caused by the absence of soil disturbance. The **presence of livestock** can boost the earthworm population by returning much of the nutrients and carbon from the grass back to the soil in the form of dung, which is a rich source of food for earthworms (Lowe & Butt, 2005), resulting in increased earthworm abundance in the presence of dung patches (Bacher et al., 2018; Curry et al., 2008). However, when stocking rates increase too much, the cattle trample the ground, thus trapping and squishing earthworms and compacting the soil, which has an adverse effect on earthworm abundance and biomass ha^{-1} (Cluzeau et al., 1992; Hoeffner, Santonja et al., 2021; King & Hutchinson, 1983). With regard to **botanical composition**, the presence of legumes was found to positively affect earthworm abundance and biomass as they offer a rich food source (Eisenhauer et al., 2009; Fischer et al., 2014). Several studies and reviews have shown that earthworms are more abundant in **fertilised grasslands** than in non-fertilised ones (Deru et al., 2018; Timmerman et al., 2006). This finding can be linked to a higher level of plant primary production on fertilised fields, which also entails additional litter production that can serve as earthworm food (Curry et al., 2008; Schon et al., 2011). In the case of organic fertiliser application in the form of stable manure, compost or bokashi, the fertiliser itself can also serve as a food

source for earthworms (Deru et al., 2023). All of the aforementioned studies have provided important insights, but to date, the effects of grassland renewal strategies on earthworm population dynamics remain understudied.

Grassland renewal involves the killing of the existing sward by herbicide application or ploughing or a combination of both. Subsequently, the grassland is reseeded, either immediately or after a temporary conversion to arable land. The renewal of grassland is considered an important management tool to maintain high productivity (Andueza et al., 2016; Iepema et al., 2020). Most Dutch dairy farmers renew their grassland every 5 to 10 years, even though a sward can in principle last for decades (Iepema et al., 2020). There are various reasons why grassland renewal is applied more frequently. For example, after a grassland has suffered from drought and part of the grass has died, it becomes susceptible to weed invasion. Grassland renewal can then be applied to re-establish a high quality sward with the desired species. Additionally, renewal can be used to boost the productivity of the grassland through the introduction of new grass cultivars (Chaves et al., 2009) and through improved rooting depth and soil structure associated with renewal.

Despite these benefits, grassland renewal sometimes has a negative impact on earthworm communities and even general soil quality (Iepema et al., 2022). Renewal is accompanied by both temporary **loss of vegetation cover** and **soil disturbance**. Especially when grassland is temporarily converted to arable land, renewal can have severe and long-lasting consequences for earthworms (Van Eekeren et al., 2008). Many studies have demonstrated that arable land harbours smaller and often less diverse earthworm populations than grasslands (Boag et al., 1997; Hoeffner, Hotte et al., 2021; Mazur-Pączka et al., 2019; Van Eekeren et al., 2008). This is linked to the decreased input of organic matter and the higher level of soil disturbance that is generally associated with arable cropping. Nevertheless, the topic of grassland renewal has received little scientific attention so far. As earthworms play a major role in grassland ecosystem functioning and may assist in climate adaptation, the effects of grassland renewal on earthworm population dynamics needs to be better understood. We aim to contribute by testing how earthworm population dynamics are affected by grassland renewal strategies with and without tillage and with and without loss of vegetation cover. This knowledge can help farmers and policy makers to make management decisions that support earthworm-mediated ecosystem services and in particular the water-regulating capacity of the soil.

1.5.4 The history and future of earthworm inoculation

When the positive contribution of earthworms to agricultural systems became better understood, an interest emerged in actively managing earthworm populations. The first reports of **intentional earthworm inoculation** date back to 1925, in New Zealand (Hamblyn & Dingwall, 1945). Earthworm inoculation was of special interest in New Zealand as the species native to this country were largely displaced when native vegetation was cleared for agricultural development (Lee, 1959). From the 1940s onwards, the New Zealand Agricultural Department conducted numerous field trials in which non-native earthworms (mainly *A. caliginosa*) were introduced with the objective of improving pasture quality. It took the earthworms some years to firmly establish a population (Stockdill, 1959), but a number of successful trials showed **productivity increases** of 28-110% (Stockdill, 1966).

In the same period, a similar experiment was carried out in the Netherlands. In 1957, the Dutch Flevopolder was created, a reclaimed land area of 970 km^2. The main aim of the reclamation project was to increase agricultural production, in answer to the rising demand for food from growing cities. Earthworms were virtually absent from these young reclaimed soils, although the conditions were favourable. In this area, dairy farmers encountered several problems with their grasslands. In the absence of decomposing organisms, a dense continuous layer of organic matter formed, suffocating the vegetation below, a process referred to as 'mat formation'. The grasslands also suffered from sward detachment, weeds, soil compaction and damage through urine scorching. In an attempt to alleviate these issues, the soil was inoculated with earthworms to speed up natural colonisation. In the years that followed, many soil characteristics improved significantly, such as infiltration capacity, water conductivity, aeration, penetration resistance, shear strength and susceptibility to botanical deterioration (Hoogerkamp et al., 1983). Earthworms fed on the grass litter, and consequently the problem of mat formation was resolved and the grasslands were found to be more productive with earthworms present.

In the 1990s, several researchers worked on earthworm inoculation. Introduction of anecic species *Aporrectodea longa* into Australian pastures seemed successful, as indicated by offspring production after 16 months (Garnsey, 1994). Baker et al. (1999) confirmed this by showing that *A. longa* had been able to establish a population in grassland, four years after inoculation in South-Eastern Australia. Stein et al. (1992) observed a slow but steady dispersal from inoculation points to a distance of 189 m of *A. caliginosa*, *A. longa* and *L. rubellus* in Dutch permanent pasture over a period of

18 years. Some years before, in Ireland, it was found that inoculation through grass sod transplantation was successful for shallow working earthworm species, but not so much for deep burrowers like *A. longa* and *L. terrestris* (Curry & Boyle, 1987). Observations by Bohlen et al. (1995) emphasised that it was maintaining populations that should be given priority, rather than impairing populations and then attempting to fix this through inoculation. These researchers found in maize fields in Ohio that it was easier to reduce the populations of *L. terrestris* and *Aporrectodea tuberculata* through electroshock than it was to increase the population by inoculation. Thus, damage control should be favoured over damage repair.

Meanwhile in the UK, Butt carried out a great deal of work on earthworm inoculations and *L. terrestris* breeding (Butt, 1993, 1998, 2008, 2011a, 2011b, 2011c; Butt et al., 1992; Lowe & Butt, 2005, 2007; Nuutinen & Butt, 1997), mainly to explore the potential of earthworm inoculation for **soil remediation**. In 2010, Butt reviewed the insights of over twenty years of research on earthworm inoculation (Butt, 2008), proving that earthworm inoculation is not straightforward but rather comes with practical challenges, ranging from unsuccessful culturing due to lack of climate control in breeding areas to unforeseen complete excavation of experimental sites by roadbuilders and to poor species selection resulting in the almost immediate death of the inoculum (Butt, 2011a). This research led to many new and valuable insights into the lifecycle of *L. terrestris*, *L. terrestris* culturing as well as strategies for earthworm inoculation. Nevertheless, the practical challenges mentioned above led to a stagnation in the exploration of earthworm inoculation as a method for soil amelioration.

Between 2011 and 2026, few scientific studies have addressed the topic of in-field earthworm inoculation. A recent meta-analysis by Petit-dit-Grézériat et al. (2024b) reports only five studies in addition to the studies presented in this thesis, namely Ampoorter et al. (2011), Forey et al. (2018), Frazão et al. (2019), Golli et al. (2015) and Ratsiatosika et al. (2021). None of these studies were carried out in temperate grassland. This lack of scientific attention is somewhat surprising because in the same period the need for climate adaptive management has only increased. As the positive effect of earthworms on soil health has been so firmly established, the path of earthworm inoculation certainly needs to be explored further. Current knowledge gaps that need to be resolved relate to the exploration of effective inoculation strategies to promote the establishment of an *L. terrestris* population in grassland on sandy soil, for example regarding the origin of the inoculum, food management

and interspecific competition. Resolving these knowledge gaps should take place preferably in field experiments and with the involvement of stakeholders.

1.6 Objectives, research questions and hypotheses

Due to climate change, precipitation patterns are shifting and the incidence of not only peak rains but also drought increases. For this reason, climate adaptive measures are crucial to ensure the continued provision of ecosystem services. The water-regulating capacity of grasslands on sandy soil has become increasingly important. Earthworms, especially deep burrowing earthworms such as *L. terrestris*, are ecosystem engineers that can alter soil hydraulic properties, thereby improving water infiltration rate and capacity. By improving the understanding of (1) habitat suitability for *L. terrestris*, (2) the effect of various grassland renewal strategies on earthworm communities and (3) the feasibility and effective methodology for *L. terrestris* inoculation, I aim to contribute to the **active promotion of earthworm populations for the sake of improving the water-regulating capacity of grasslands on sandy soil**. To tackle the current knowledge gaps regarding this topic (introduced in sections 1.5.2-1.5.4), a field inventory and various mesocosm experiments at dairy farm grasslands were executed and the following research questions were addressed:

General research questions and hypotheses:

1. **RQ** How do factors such as soil texture, groundwater level, grassland age and interspecific competition influence the presence and abundance of *L. terrestris* in grasslands on sandy soils? **H** We expect that the abundance of *L. terrestris* correlates positively with loam and clay content of the soil, as coarse-textured soils may be too prone to drought. Moreover, we expect to find greater *L. terrestris* abundance in pastures with a low groundwater table, as this reduces the risk of burrow inundation. We expect grassland age to correlate positively to *L. terrestris* presence and abundance as soil disturbance may be detrimental. Furthermore, we expect interspecific competition with species in the same feeding niche to negatively affect *L. terrestris* presence and abundance (Chapter 2).

2. **RQ** What is the effect of grassland renewal strategies that include tillage and/or loss of vegetation cover on the resident earthworm population in general and on *L. terrestris* in particular? **H** We hypothesise that both soil disturbance and soil bareness decrease earthworm abundance, as the first

can physically injure earthworms and destroy their burrows, and the latter leaves them without food source and protective layer. *L. terrestris* is likely to show a strong negative response and recover slowly, as it is dependent on a stable environment and has a slow reproduction cycle (Chapter 3).

3. We explore the feasibility of *L. terrestris* inoculation in grasslands on sandy soils that do not have a native population through the following questions: **RQ (1)** Is *L. terrestris* able to persist and produce offspring after inoculation? and **(2)** Which factors affect the colonisation success of inoculated *L. terrestris*? **H (1)** We hypothesise that if a grassland is suitable for *L. terrestris*, successful inoculation is possible, meaning that the introduced earthworms will survive, settle and reproduce. **H (2)** We further hypothesise that interspecific competition with the resident earthworm population and the origin of the inoculum influences the inoculation success (Chapters 4 & 5).

1.7 Outline of this thesis

The research questions are addressed in the following four chapters:

Chapter 2: *Lumbricus terrestris* abundance in grasslands on sandy soils in relation to soil texture, hydrology and earthworm community

We aim to elucidate *L. terrestris* habitat preferences by exploring *L. terrestris* presence and abundance in relation to various environmental factors on sandy soils, namely soil texture, groundwater level, abundance of epigeic earthworm species *L. rubellus* and grassland age. It is currently not well understood what determines the presence and abundance of *L. terrestris*. However, this question is of interest to farmers and waterboards, as the burrowing activity of *L. terrestris* may contribute substantially to the water-regulating capacity of grasslands. A systematic earthworm inventory on pastures at Dutch dairy farms in the province of Brabant was executed to collect data.

Published in *European Journal of Soil Biology* (2023)

Chapter 3: Earthworm communities are more affected by tillage than soil cover loss in an on-farm mesocosm-study in grassland

This chapter examines the effect on the indigenous earthworm community of different management practices that are related to grassland renewal. An in-field mesocosm experiment was conducted to investigate how the application of tillage and the presence or absence of vegetation cover affect earthworm abundance, demography and species composition.

Submitted to *Applied Soil Ecology* (October 2026)

Chapter 4: The anecic earthworm *Lumbricus terrestris* can persist after introduction into permanent grassland on sandy soil

This chapter explores the feasibility of grassland inoculation with *L. terrestris* earthworms. Potentially, *L. terrestris* inoculation could be used as an ecologically innovative measure to improve the water-regulating capacity of grasslands. The deep and vertical burrows created by this species improve the water infiltration rate and capacity of the soil. A mesocosm set-up at two Dutch dairy farms on pastures where *L. terrestris* did not occur was used to test whether the earthworms would survive and reproduce upon inoculation.

Published in *European Journal of Soil Biology* (2023)

Chapter 5: The effect of earthworm origin on the colonisation success of anecic earthworm *Lumbricus terrestris* (L.) in mesocosms on pasture

Building upon the insights we gained in the research described in Chapters 2 and 4, we designed a second inoculation experiment. This mesocosm experiment was conducted at two different Dutch dairy farms. We introduced *L. terrestris* of two different origins: locally collected and commercially purchased (imported from Canada). The feeding regime was altered and one of the selected farms already hosted an *L. terrestris* community.

Published in *Plant & Soil* (2025)


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Chapter 2

Lumbricus terrestris abundance in grasslands on sandy soils in relation to soil texture, hydrology and earthworm community

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Abstract

Deep-burrowing (anecic) earthworm *Lumbricus terrestris* contributes to the crucial ecosystem service of water regulation. Their deep, vertical burrows facilitate water flow and deeper rooting, the former supporting the prevention of flooding and waterlogging, the latter improving drought tolerance. In Europe, these earthworms occur in agricultural grasslands on various soil types. However, their distribution pattern can be very heterogeneous. There is no conclusive set of soil biotic or abiotic factors that determines whether *L. terrestris* occurs or not. Through a better understanding of the *L. terrestris* distribution patterns we hope to gain more insight into their potential for climate adaptive water regulation.

We executed a field inventory (n = 62) to assess the relationship between *L. terrestris* population density in grassland on sandy soils and soil silt content (loaminess), gley depth, epigeic earthworm population density and grassland age.

We found positive correlations between soil silt concentrations and *L. terrestris* population densities. Gley depth slightly correlated with population density when presented in a model with silt concentration as a predictor. Presence and population density of *L. terrestris* correlated negatively with *Lumbricus rubellus* abundance. The number of years without mechanical soil disturbance and *L. terrestris* population density were not significantly related. Unexpectedly, we found *L. terrestris* in some very sandy soils. Our data was fitted into an existing predictive model based on land use and texture (by Lindahl et al., 2009), yielding 63% accuracy. Overall, this correlative study provides further insights into *L. terrestris* habitat selection, which helps us understand the species' potential for water regulation in the widespread grassland agro-ecosystems.

Keywords: Lumbricidae, deep-burrowing earthworms, ecosystem functioning, grassland, ecosystem engineers, water regulation

1. Introduction

Global climate changes are characterised by prolonged dry periods and intensified peak rainfall (Pachauri et al., 2014; Orlowsky & Seneviratne, 2012). Although the former has gained more attention until now, the latter could also entail major impacts on plant growth, biogeochemical cycles and nutrient losses (Andriuzzi et al., 2015; Beier et al., 2012). For example, flooding and waterlogging on grasslands reduces oxygen diffusion in soils, thus depriving plant roots of adequate oxygen supply. The resulting oxygen deficiency (hypoxia) or even completely anaerobic conditions (anoxia) in the rhizosphere enhance N_2O emissions and inhibit nutrient uptake and, thus, plant growth (Reyer et al., 2013; Sairam et al., 2008). Additionally, changing precipitation patterns cause societal problems, including damage to buildings and infrastructure in the case of flooding, but also leads to water shortages (Ciscar et al., 2011; Wang et al., 2016).

As ecosystem engineers, earthworms provide various ecosystem services (Van Groeningen et al., 2014). In permanent grasslands, where mechanical soil disturbance is low (e.g. no chisel ploughing), earthworms play an important role in soil bioturbation and thus improve water regulation (Deru et al., 2018). Deep-burrowing earthworms (*anecics*, sensu Bouché, 1977) such as *Lumbricus terrestris* and *Apporectodea* longa play an important role in the ecosystem service of water regulation (Andriuzzi et al., 2015; Capowiez, Cadoux, Bouchant et al., 2009; Stockdill & Cossens, 1966). They create vertical, semi-permanent burrows, reaching depths up to 2 meters, thus enhancing soil macroporosity. This behaviour increases both the infiltration rate and capacity of the soil and avoids waterlogging and flooding (Blouin et al., 2013; Brown et al., 2004; Shipitalo & Le Bayon, 2004; Nuutinen & Butt, 2002; Jones et al., 1994; Lee, 1985). Furthermore, earthworm activity increases rooting space, which is beneficial for plant growth and promotes drought tolerance (Edwards & Lofty, 1980; Stockdill & Cossens, 1966). Several factors are known to play a role in facilitating the presence of anecic earthworms:

1. Soil texture: Positive interactions have been found between anecic earthworm density and soil silt and clay content (Lee, 1985; Guild, 1948; Nieminen et al., 2011; Nordstrom & Rundgren, 2023; Valckx et al., 2009; Whalen & Costa, 2009). Guild (1948) found the highest population densities of *L. terrestris* and *A. longa* adults under permanent pastures in alluvial and loamy soils, respectively. Furthermore, within-field heterogeneity in terms of soil clay contents

(> 8%) was found to correlate with A. longa found in higher densities in the more clayey parts (Valckx et al., 2009). Nordström and Rundgren (2023) suggested close positive interrelations between soil clay content and other soil factors stimulating earthworm population establishment, such as organic matter content and water retention. Hawkins et al. (2008) used a soil column experiment to show that sand layers complicate *L. terrestris* burrowing activity, which is assumed to relate to the abrasive coarse texture of sandy soils compared to loamy soils. Both susceptibility to drought in soils with low clay contents and compaction and waterlogging in heavy clay soils could be detrimental to earthworms in general (Lee, 1985; Lindahl et al., 2009; Nordstrom & Rundgren, 2023) and *L. terrestris* in particular (Lindahl et al., 2009).

2. Ground water levels: Very few studies assessed whether groundwater levels (wholly or partly) define anecic earthworm population density and its corresponding biomass. Decaëns et al. (2003) observed lower anecic earthworm densities in both periodically flooded sandy soils (fluviosols) and soils with peat layers (histosols) than in more clayey plateaus. This finding may indicate that these earthworms escape environments with periodic water excess. At the field scale, Nuutinen et al. (2001) found higher *L. terrestris* burrow and population densities above tile subdrains located at 1-meter depth in sandy clay soil. This finding could either mean that reproduction rates are higher in these positions or that *L. terrestris* is able to select a suitable habitat whilst moving over the soil surface (Nuutinen et al., 2011). Although current research is inconclusive, favourable soil hydrological conditions are likely to play a role in successful anecic earthworm settlement (Nuutinen et al., 2011; Valckx et al., 2011).

3. Interspecific competition: Decaëns et al. (2008) concluded that local earthworm species assemblies are highly structured by interspecific competition. Under lab conditions, Butt (1998) showed that *L. terrestris* growth rate was impaired through interspecific competition, which is probably related to resource depletion. According to Butt (1998), a larger species like *L. terrestris* cannot keep up with smaller epigeic earthworms that have the advantage of a higher growth and reproduction rate. *Lumbricus rubellus* is an epigeic earthworm species that can be abundant in grassland and, in Europe, is often found to co-occur with *L. terrestris*. As *L. terrestris* and this epigeic species both feed on plant litter, interspecific competition for resources seems plausible.

4. Management: Soil physicochemical factors and groundwater levels could set limits for anecic earthworm population density, and thus field management could define local limitations (Decaëns et al., 2003; Nieminen et al., 2011). Anecic earthworms have been found to prefer low soil disturbance conditions (e.g. no ploughing), which are characteristic of orchards, perennial cropland, permanent grasslands as well as field margins (Decaëns et al., 2003; Lee, 1985; Lees et al., 2016; Low, 2016; Nieminen et al., 2011; Van Eekeren et al., 2008). Population density recovery in grasslands after mechanical soil loosening may take at least two to three years (Lees et al., 2016; Van Eekeren et al., 2008; Watkin & Wheeler, 1996). Soil compaction has also been shown to impede burrow formation and maintenance by *L. terrestris* (Beylich et al., 2010; Capowiez, Cadoux, Bouchand et al., 2009). Benefits of high-quality (high N) plant litter and organic fertilizer inputs for population growth of anecic earthworms have frequently been demonstrated (Andersen, 1980; Brown et al., 2000; Lee, 1985; Low, 2016; Lowe & Butt, 2005; Riley et al., 2008; Watkin & Wheeler, 1996). The rate of high-quality soil organic matter input is therefore an important determinant of anecic earthworm abundance.

Currently it is known that the distribution of *L. terrestris* is heterogeneous at the field and landscape scale. However we lack a set of parameters that explains their occurrence especially on grasslands on sandy soils, while the ecosystem service of water regulation in this land use and soil type combination is very important. This field inventory aims to improve our understanding of the factors that define *L. terrestris* presence and abundance in grasslands on sandy soil. Factors of importance are expected to be (1) soil texture characteristics, (2) groundwater level, (3) competitive interaction with resident earthworm species and (4) land use and management.

We hypothesised that anecic earthworm population densities are (1) positively correlated with soil silt concentration, (2) positively correlated with the depth of the highest groundwater level, (3) negatively correlated with epigeic earthworm abundance, and (4) positively correlated with the number of years without mechanical soil disturbance.

Table 2.1 Categorisation of sampling points (number of sampling points between brackets) by soil type, soil texture class, landscape geomorphology, ground water stage (based on highest [HGL] and lowest [LGL] groundwater levels throughout the season) and grassland age

Soil type[1]		**Soil texture class**[2]		**Geo-morphology**	**Groundwater stage**		**Grassland age**
	A-horizon		*Texture (%)*		*HGL (cm)*	*LGL (cm)*	
Sandy thick earth soil (19)	≥50 cm	Loamy sand (43)	Sand: 50-82.5; Clay: ≤8; Silt: 17.5-50	Plateau (26)	<25 (13)	120-180 (13)	≤3 years (22)
Sandy hydro-earth soil (31)	<50 cm	Light loamy sand (13)	Sand: 82.5-90; Clay: ≤8; Silt: 10-17.5	Plain (34)	<25 (6)	>180 (6)	>3 years (40)
Sandy xero-earth soil (12)	<50 cm	Loam-poor sand (6)	Sand: 90-100; Clay: ≤8; Silt: 0-10	Slope (2)	40-80 (13)	>180 (13)	
					80-140 (12)	>180 (12)	
					>140 (18)	140-180, >180 (18)	

[1] Van der Veer, 2006; [2] Schils, 2012, Van der Meulen et al., 2006.

2. Materials & methods

2.1 Study area

Earthworm and soil sampling was carried out between March and May 2021 in an area with sandy soils south of the National Park Loonse and Drunense Duinen between Den Bosch and Tilburg in the province of Noord-Brabant, the Netherlands. This area was selected since it has a variety of sandy soils with different textures and is known to host *L.terrestris* (Linnaeus, 1758). The research area is a patchy landscape where small woodlands alternate with grassland, arable land and orchards, and it is heterogeneous in terms of its geomorphology, groundwater levels and soil texture. Geodata (PDOK Viewer, accessed March 2021; Knotters et al., 2018) was used to find grasslands of varying geomorphology interrelated with a range of soil types, texture classes and ground water stages (Table 2.1, Table 2.2). Grasslands were divided into age categories 'young' (≤ 3 years) and 'old' (> 3 years), based on the number of years without disturbance (Table 2.1). The 'disturbance' was either direct grassland renewal in the form of ploughing followed by reseeding (grassland to grassland) or in the form of ploughing and seeding grassland after maize cultivation (maize to grassland). Ultimately, 31 grasslands that were distributed over 11 farms complied with the selection criteria. Because of the multiple selection criteria it was not possible to create a completely balanced set of grasslands with regard to age (11 young grasslands and 20 old grasslands).

Weekly rainfall during the earthworm and soil sampling period was higher in March 2021 than in April 2021 (Figure 2.1).

Table 2.2 Range of soil physico-chemical parameter values measured in the sampled grasslands, at 0-10 cm and 30-40 cm depth; mineral percentages are calculated as fraction of total mineral weight; SOM percentages are calculated as fraction of total soil weight

	0-10 cm depth			30-40 cm depth		
	Min.	Max.	Mean	Min.	Max.	Mean
Clay (%)	1	9	3	1	10	3
Silt (%)	7	42	25	7	42	24
Sand (%)	52	91	71	50	92	70
SOM (%)	2.5	16.0	5.2	1.1	7.4	2.6
pH-KCl	4.5	6.9	5.4	4.6	7.4	5.4

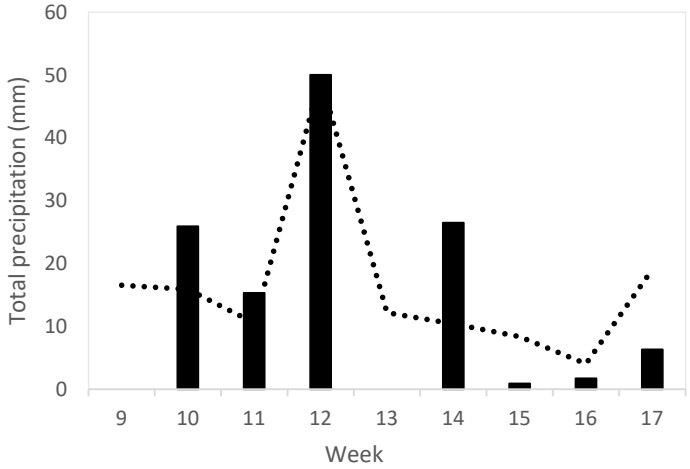

Figure 2.1 Bars indicate total precipitation per week measured at Tilburg weather station in March and April 2021 (KNMI, n.d.). Trendline (dots) represents the 10-year precipitation average (2011-2020).

2.2 Earthworm sampling and burrow counting

In each grassland, two plots (40x40 cm) were sampled (62 plots in total). The plots were located at representative locations at least 40 m apart from each other, distance to the border of the grassland was at least 10 m. We applied a combination of hand-sorting and AITC extraction (Pelosi et al., 2009). From each plot, one cube of 20x20x20 cm was dug out and transported to the laboratory. The cube was then hand-sorted in order to identify all the earthworm species that it contained and to count their total numbers. Three more 20x20x20 cubes were dug out in order to create a pit of 20 cm deep and 40x40 cm in length and width. At the bottom of the 40x40 cm pit, the holes were counted that were assumed to be burrows of *L. terrestris* (ø > 2 mm), to check for a correlation with *L. terrestris* abundance. Subsequently, approximately 4 litres of 0.01% allyl-isothiocyanate (AITC) solution was applied to the bottom of the pit to collect *L. terrestris* from the deeper soil layers. All earthworms emerging at the pit surface within 20 minutes were collected, rinsed with water and stored in containers. The earthworms collected by hand-sorting and AITC extraction were washed with water, dried, weighed and stored in ethanol at 7°C until further species identification was carried out. Earthworms were categorized as 'adult' if a clitellum was present and as 'juvenile' if a clitellum was absent. Adults were identified to species level (Sims & Gerard, 1985), for juveniles it was checked whether they were *L. terrestris*. Juveniles of *L. rubellus* and *L. terrestris* were distinguished from each other by checking considerable thickenings of any septa, which, as described by Stöp-

Bowitz, are absent for *L. rubellus* (Stöp-Bowitz, 1969). No other species that could have been interchanged with *L. rubellus* or *L. terrestris* were present in the samples.

2.3 Assessing soil profile and soil sampling

An auger (10 cm diameter) was used to assess the soil profile (0-120 cm) and the soil type of each earthworm sampling plot. Information was recorded on A-horizon thickness, depth of clay layers and hydromorphic characteristics. Based on the results of texture analysis, soils were categorised as loam-poor, light loamy or loamy sands, according to a Dutch texture classification (Schils, 2012; Van der Meulen et al., 2006) (Table 2.1). Furthermore, they were classified as Earth soils (Fimic Anthrosols/gleysols), and most were characterised by an anthropogenic A-horizon, with or without the formation of hydromorphic features (Van der Veer, 2006) (Table 2.1). Moreover, gley depth in cm was recorded as a measure for temporary maximum groundwater or pseudo-groundwater tables. Gley layers are characterised by a pattern of rust spots resulting from seasonal water table fluctuations.

Field-moist soil samples were taken from the 0-10 and 30-40 cm soil layer with a gouge auger (2.5 cm diameter) from 15 spots within a 2-metre radius from the earthworm sampling plot to realise composite samples of approximately 200 g. Soil samples were stored at 7°C until further analysis. Samples were analysed at Eurofins Wageningen for soil physical and organo-chemical characteristics. Clay (< 2 µm diameter) content were determined through density fractionation (NEN 5753, 2018). Soil clay, silt and sand content was determined by the pipette method and sieving (resp. fractions < 2, 2-50 and > 50 µm) and calculated as a fraction of mineral soil weight. Soil organic matter (SOM) was determined by loss-on-ignition (Ball, 1964): after drying at 105 ± 5°C, the soil sample was ignited at 550 ± 25°C after which soil organic matter (SOM) was calculated as a fraction of the 105°C dried soil. Soil acidity (pH) was measured after extraction in a 0.01 M $CaCl_2$ solution (Table 2.2).

2.4 Grassland management

Data about the history of land use (grassland age and prior crop to grassland) and fertilisation (e.g. type of organic manure applied – slurry or solid) were obtained from the farmer.

2.5 Data analysis

Prior to data analysis, earthworm abundance data was converted to number of individuals m^{-2}. For *L. terrestris*:

$$Individuals\ \left(\frac{\#}{m^2}\right) = Hand\ sorted\ cube * \left(\frac{1}{0.2 * 0.2}\right)(m^2) + AITC * \left(\frac{1}{0.4 * 0.4}\right)(m^2)$$

Here, hand-sorted cube and AITC are the number of individuals retrieved with the methods described in section 2.2. The number of epigeics and endogeics retrieved from the hand-sorted cube in the laboratory were also converted to number of individuals m^{-2}. The number of burrows counted in the 40x40 cm pit was also converted to burrow density m^{-2}.

For all statistical analyses in this research, R version 4.0.3 (2020-10-10) and RStudio version 1.2.5042 (Rs Team, 2020; R Core Team, 2020; RStudio Team, 2020) were used. The correlation between soil type, texture class, groundwater stage and grassland age categories (Table 2.1) and *L. terrestris* population density was analysed with the lme4 package (Bates et al., 2009). Linear mixed models were created in which the categorical data was used as a fixed effect and *farm* and *field* were used as random factors. Chi-square and Fisher's exact test were applied to assess the correlation between different factors and the absence/presence of *L. terrestris*. Bonferroni corrections were applied when performing multiple comparisons.

For all correlative analyses, all 62 data points with and without *L. terrestris* were used together. A general selection of variables correlating with *L. terrestris* population densities was retrieved by matrix-wise Pearson's r correlations of all the soil and earthworm community variables measured. Further selection candidate correlative variables took place by conducting stepwise Akaike's Information Criterion (AIC) forward selection. Variables that correlated significantly with *L. terrestris* population densities were used to perform a Principal Component Analysis (PCA) with the stats package (R Core Team, 2020) to ordinate *L. terrestris* population densities in a multidimensional space.

Furthermore, these selected variables were used for the creation of linear models to explore further the correlation with total, adult and juvenile densities of *L. terrestris*. Of each model presented, the coefficient of determination (R^2), $R^2_{adjusted}$ and the Root Mean Square Error (RMSE) are presented, along with significance levels of each variable within the model. Collinearity within the models was considered not to be an issue when the Variance Inflation Factor (VIF) values were < 10. Additional

correlation analyses were performed between *L. terrestris* density and burrow counts m^{-2}.

Normal distribution of linear model and mixed-model residuals was checked with the Shapiro-Wilk test and visually with histograms, quantile-quantile-plots (QQ), boxplots and residual plots. Log- and squared- and square root-transformations were applied if necessary for meeting the normality assumptions and performing regression analyses. When statistical analysis was performed on transformed data, this is mentioned in the figure and table captions. When normality assumptions could not be achieved for categorical data, the Kruskal-Wallis H test was applied if the assumption of equal variance (Levene's test) had been met.

The data, as obtained from the field inventory, was fitted into the existing classification tree for *L. terrestris* density m^{-2} as created by Lindahl et al. (2009). In accordance with Lindahl et al. (2009), 'land-use type' was classified as 'perennial'. The percentage of lutum, sand and silt from the soil samples at 10 cm depth and 40 cm depth was used to determine the USDA soil texture classification of each sample. The obtained soil classifications were then translated into the categories (fine, medium, coarse) used by Lindahl et al. (2009).

3. Results

3.1 General overview

Figure 2.2 shows a general overview of the significant correlations between the *L. terrestris* population density and the parameters measured. *L. terrestris* population density (*TotalLT*) correlated with soil silt content at 30-40 cm depth and the presence of the epigeic species *L. rubellus*. Soil clay content and gley depth very slightly contributed to explaining the variation in *L. terrestris* population densities. Non-correlating factors (soil type, texture class, depth of A-horizon, groundwater stage, pH and SOM) were left out of the PCA for clarity (See Suppl. Tables S2.5A and S2.5B for an overview of all tested correlations).

3.2 Soil abiotic characteristics and *L. terrestris* population density

Total, adult and juvenile *L. terrestris* densities did not differ significantly between soil texture classes. A range of 0-441 *L. terrestris* individuals m^{-2} was found in loamy sands, compared to 0-144 individuals m^{-2} in light loamy sands and 0-105 individuals

m^{-2} in loam-poor sand (Table 2.3). Both adult and juvenile *L. terrestris* mean densities were highest in the loamy sand soil category (Table 2.3).

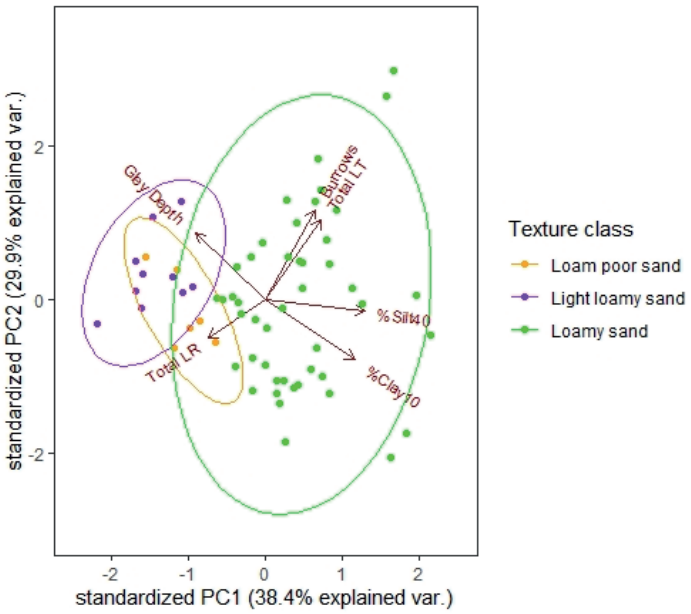

Figure 2.2 Principal Component Analysis: ordination of sampling points in the plane shown along multiple soil textural and earthworm population correlation vectors, defined by Principal Components (PC) 1 and 2. Gley Depth = depth (cm) at which gley features start; burrows = number of vertical anecic earthworm burrows m^{-2}; Total LT = total *Lumbricus terrestris* (anecic) individuals m^{-2}; Total LR = total *Lumbricus rubellus* (epigeic) individuals m^{-2}; %Silt40 = percentage of silt in the soil at 40 cm depth; %Clay10 = percentage of clay in the soil at 10 cm depth.

Table 2.3 *Lumbricus terrestris* total, juvenile and adult number of individuals per texture class (# m^{-2}). In the 'Texture class' column, the number of sampling points is indicated between brackets. In the 'Mean' column, the standard deviation (SD) is indicated between brackets. Statistical analysis was applied to square root transformed data.

Lumbricus terrestris	**Texture class (n)**	**Mean (SD)**	**p-value**
Total	Loam-poor sand (6)	17 (43)	0.48
	Light loamy sand (10)	21 (48)	
	Loamy sand (46)	65 (97)	
Adults	Loam-poor sand (6)	1 (2)	0.28
	Light loamy sand (10)	6 (14)	
	Loamy sand (46)	14 (16)	
Juveniles	Loam-poor sand (6)	17 (41)	0.56
	Light loamy sand (10)	15 (39)	
	Loamy sand (46)	51 (88)	

Silt (*%Silt40*) and clay (*%Clay10*) concentrations at a soil depth of 30-40 cm and 0-10 cm, respectively, were found to correlate significantly with *L. terrestris* total and adult population densities. Silt concentration correlated with *L. terrestris* total ($R^2 = 0.21$; $p < 0.001$) as well as adult ($R^2 = 0.33$; $p < 0.025$) and juvenile ($R^2 = 0.15$; $p < 0.025$) densities (Suppl. Table S2.1). Clay concentration was only found to correlate significantly with total, adult and juvenile *L. terrestris* population densities as an added variable to silt concentration (Suppl. Table S2.1).

Total population densities of *L. terrestris* were found to correlate positively with soil silt concentration at 30-40 cm and negatively with soil clay concentration at 0-10 cm depth (Figure 2.3). Within the loamy sands, a strong positive trend was observed in *L. terrestris* population densities between a soil silt percentage of 20% and 40% (Figure 2.3).

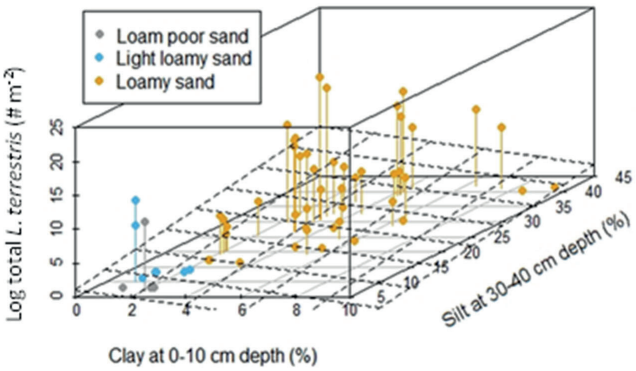

Figure 2.3 Correlation between soil silt and clay concentration and total *Lumbricus terrestris* population densities m^{-2}. The plane represents the related linear model (Suppl. Table S2.1). Texture classes based on a Dutch texture triangle (Schils, 2012; Van der Meulen et al., 2006). Total *L. terrestris* population densities are log transformed.

The results suggest that higher clay concentrations arising at 0-10 cm depth in loamy sands correlate with lower *L. terrestris* population densities. However, this correlation was only significant when modelled together with the soil silt concentration.

For 63% of the samples, fitting our data on texture into the classification tree by Lindahl et al. (2009) resulted in an accurate estimation of *L. terrestris* density (low, medium, high; < 3, 3-10, > 10 m^{-2}, respectively). The accuracy of the classification tree for medium-textured soils was 51%, for coarsely-textured soils, it was 69%.

3.3 Gley depth and *L. terrestris* population density

Gley depth measured on site positively correlated with *L. terrestris* total densities when presented in a model with the silt concentration predictor ($R^2 = 0.25$; $p < 0.05$) (Suppl. Table S2.2), Figure 2.4 confirms this. Figure 2.4 shows also that *L. terrestris* population densities tend to be slightly higher at the sample locations where gley was found deeper in the soil profile. It should be noted that gley depth correlated negatively with both silt and clay concentrations at 10 and 40 cm soil depth ($p < 0.05$) (Suppl. Table S2.5A and S2.5B).

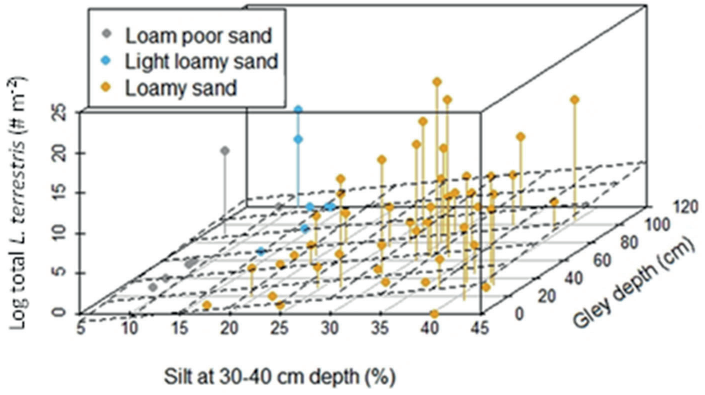

Figure 2.4 Correlation between soil silt concentration at 30-40 cm depth, gley depth and total *Lumbricus terrestris* population densities m^{-2}. The plane represents the related linear model (Suppl. Table S2.2); StartGley is the depth (cm) at which the gley starts. Texture classes based on a Dutch texture triangle (Schils, 2012; Van der Meulen et al., 2006). Total *L. terrestris* population densities are square root transformed.

3.4 Correlation between *L. terrestris* and *L. rubellus* population density

A significant negative correlation was observed between *L. rubellus* population densities and *L. terrestris* total and adult population densities ($R^2 = 0.10$; $p < 0.025$ and $R^2 = 0.11$; $p < 0.025$ respectively) (Suppl. Table S2.3). No significant correlations were found between *L. rubellus* population densities and *L. terrestris* juvenile population densities. The significant correlations are presented both in models with only *L. rubellus* population density as a factor and in models where soil silt concentration and gley depth are added as factors (Suppl. Table S2.3).

A significant negative correlation was found between silt concentration and adult *L. rubellus* population density ($R^2 = 0.10$; $p < 0.025$). Mean *L. rubellus* total population densities were 79, 133 and 45 individuals m^{-2} in loam-poor, light loamy and loamy sands, respectively.

3.5 Grassland age and *L. terrestris* population density

Grassland age did not significantly correlate with *L. terrestris* population densities (Suppl. Table S2.5A and S2.5B). Furthermore, differences in *L. terrestris* population densities between grasslands classified as older (> 3 years) and younger (< 3 years) were not significantly different.

3.6 Correlation between *L. terrestris* burrow density, soil texture and *L. rubellus* population density

The density of vertical burrows, which were assumed to be (formerly) inhabited by *L. terrestris* individuals, was linearly correlated with the total *L. terrestris* population density (Figure 2.5). Burrow density also correlated with the factors discussed in the previous sections: it correlated positively with silt concentration and gley depth and negatively with clay concentration and *L. rubellus* population density (Suppl. Table S2.4).

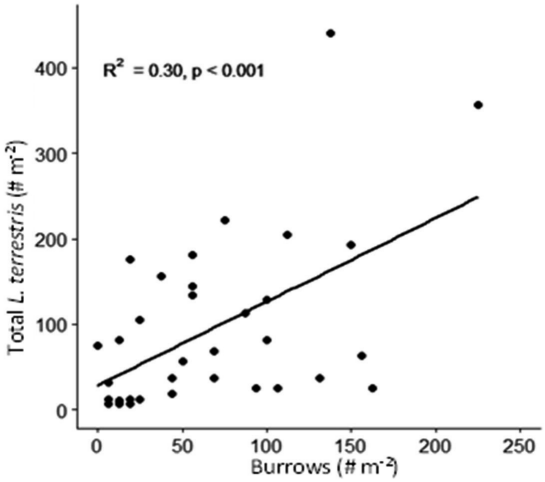

Figure 2.5 Correlation between total *Lumbricus terrestris* population density and vertical burrows counted at the same sample site.

4. Discussion

4.1 Texture

Soil silt concentrations at 30 to 40 cm depth positively correlated with both *L. terrestris* adult and juvenile densities in grasslands. This finding supports our hypothesis that loamier grassland soils host higher *L. terrestris* population densities. Clay content at 0 to 10 cm depth correlated negatively with *L. terrestris* population densities (Table

2.2; Figure 2.3). Moreover, *L. terrestris* was also present in some very sandy soils (Figure 2.3; Table 2.3), which was not in line with our expectations.

Higher densities of *L. terrestris* in loamier (less sandy) soils were found in earlier research (Decaëns et al., 2003; Guild, 1948). Hawkins et al. (2008) and Lee et al. (1985) suggested that earthworm survival is impeded by the coarse texture and drought proneness of sandy soils. However, Guild (1948) did find *L. terrestris* in coarsely textured soil, albeit in lower densities than in medium-textured soils. So did Chamberlain and Butt (2008) during their inventory in a sand dune ecosystem in NW England, they reported low numbers of *L. terrestris* at a car park with sandy soil and low organic matter content (0.9%). Apparently, *L. terrestris* can also survive in loam poor sand, as was confirmed by our finding of *L. terrestris* in very sandy soil on one of the farms.

It may instead be that the *indirect* advantages of finer textured, loamy sand play a role in facilitating higher *L. terrestris* densities: these could create a niche with preferable water regulation compared to coarser textured soils (Holmstrup et al., 2011; Curry, 2004). Better moisture and nutrient retention in loamy sand has been suggested to provide a more favourable environment for earthworms than loam poor sand (Lee, 1985; Nordstrom & Rundgren, 2023; Ouellet et al., 2008). Furthermore, loamy sands are less sensitive to compaction and partly anaerobic conditions due to waterlogging than soils that are higher in silt and clay content (Diaz-Zorita & Grosso, 2000; Smith et al., 1997) and badly drained (Rachel Creamer, pers. comm., 2021). This could also favour better *L. terrestris* settlement, despite the coarser soil texture.

In recent studies, soil compaction has been related to more superficial burrowing by *L. terrestris* (Ducasse et al., 2021), which indicates the plasticity of their behaviour. As a result, macroporosity in compacted soils is decreased (Pöhlitz et al., 2019). Soil texture and its susceptibility to compaction could strongly affect water regulation, therefore we think they are very important factors in *L. terrestris* settlement in more sandy soils.

Figure 2.5 shows the correlation between the number of burrows and the number of *L. terrestris* found is positive ($R^2 = 0.30$). *L. terrestris* is known to create burrows that sometimes – not always (Pitkänen & Nuutinen, 1997; Bastardie et al., 2003) – branch below the soil surface (Capowiez et al., 2015; Butt et al., 2014), which could have resulted in varying numbers of *L. terrestris* per hole counted. Additionally, burrows can survive longer than their inhabitants (Potvin & Lilleskov, 2017). For this reason it

was decided not to discuss burrow abundance in relation to the measured variables. We consider burrow counting a useful quick scan method for assessing *L. terrestris* presence and activity in the field. However, the method does not generate reliable data about the current actual *L. terrestris* population density.

4.2 Predictive power of a land use and texture model

Although *L. terrestris* population density was not significantly related to soil texture class, when fitting our population density data into the Lindahl et al. (2009) land use and texture model, we found an overall accuracy of 63%. In medium-textured soils, the accuracy of the model was 51%; in coarsely textured soils, the accuracy of the model was 69%. Eight of our samples (out of a total of 62) could not be fitted into the model as their difference in texture between 10 cm depth and 40 cm depth did not allow us to assign them to the texture classes that were defined by Lindahl et al. (2009). They report a 71% accuracy of their classification tree for predicting *L. terrestris* density based on land use and soil texture.

The accuracy of the classification tree appeared limited in medium-textured soils for our data, we present three possible explanations. First, in the Lindahl et al. (2009) paper, the classification tree is correct in for fine and medium textured soils in 90% of the cases. It could be that in coarsely-textured soils, it is mainly texture or effects of texture that prevent high *L. terrestris* densities, whereas in medium-textured soils, soil texture and its direct implications are not paramount. Second, we think that the textural classes that were used in our inventory may have been too broad to bring to light existing associations between earthworms and soil texture. This might explain why we did not find significant correlations between texture classes and *L. terrestris* population density. We recommend to increase the resolution of soil textural classes in future earthworm research. Third, the addition of an extra predictor after soil texture could possibly improve the predictive power of the classification tree. However, it is difficult to determine which parameter qualifies. We found that gley levels and grassland age do not explain enough variation. Holmstrup et al. (2011) tried to find associations between soil texture, soil water characteristics and earthworm populations. They found that none of the investigated parameters explained the earthworm population, the parameters being: texture, pH, bulk density, water holding capacity and total C. Possibly the addition of a step regarding soil water regulation through the soil profile (e.g. water levels, infiltration capacity, penetration resistance) could improve the predictive power of the classification tree.

4.3 Gley levels

Gley depth was found to have low predictive power for *L. terrestris* total population densities, and only when added to models with soil silt concentration as a predictor (Figure 2.4; Suppl. Table S2.2). As gley depth is an indicator of the highest groundwater levels, we expected this correlation to be stronger.

The absence of compaction layers prone to waterlogging and associated formation of temporal shallow 'groundwater' levels (pseudo-groundwater) indicates well-structured soils. Previous research suggests that soils that are well-structured, porous and deep-drained soils are suitable for anecic earthworms (Capowiez, Cadoux, Bouchand et al., 2009; Decaëns et al., 2003; Nuutinen et al., 2001; Valckx et al., 2011; Hoekstra et al., 2021). In deeper soil layers, anecics possibly avoid predation, highly fluctuating soil moisture and temperatures in the top layers.

To better understand the relationship between *L. terrestris* population density and groundwater dynamics, we recommend researchers in the future measure both the highest and lowest soil groundwater levels as well as subsoil bulk density to assess susceptibility to waterlogging, which could affect *L. terrestris* population densities.

4.4 Earthworm species interactions

We hypothesised a negative correlation between *L. rubellus* abundance and *L. terrestris* abundance, and the results confirmed this. Both these species feed on surface organic material; therefore, interspecific competition between these species may arise when food sources are limited. This could have contributed to the negative correlation between *L. terrestris* and *L. rubellus* population densities. *L. rubellus* has a higher reproduction and growth rate than *L. terrestris* and may therefore outcompete the latter for limited aboveground food sources (Butt, 1998; Uvarov, 2009).

Negative interactions between the two species were already suggested in previous research under semi-controlled conditions but not yet in a field inventory like ours. Lowe and Butt (2002) found lower growth rates of *L. terrestris* individuals when grown together with *L. rubellus* adults than when grown together with *L. terrestris* adults. Laboratory experiments by Eriksen-Hamel and Whalen (2007) indicated that heavier earthworms (like *L. terrestris*) show a stronger reduction in growth rate than smaller earthworms in a multispecies setting. Van de Logt (submitted, 2023) observed a negative correlation between *L. rubellus* population density and *L. terrestris* population density when both species were present in a mesocosm set-up.

Differences in habitat preferences of anecics and epigeics may also have played a role in the negative correlation observed between *L. terrestris* and *L. rubellus* population densities in sandy grasslands. Decaëns et al. (2003) observed dominance of epigeics and endogeics in low-lying soils (histosols, fluviosols) with periodic water excess, while anecics were more prevalent in higher-located, better-drained soils (reductisols). They indicate that land use and specific soil properties may also explain the distribution of earthworm ecological groups (e.g. peat layer in histosols and sandy texture of fluviosols) (Decaëns et al., 2003). In a modelling study based on empirical data by Palm et al. (2013), it was suggested that epigeics prefer lower elevations, in contrast with anecics. This suggestion was based on the deep-burrowing activity of anecics, avoiding high groundwater levels, and the preference of epigeics for moist soils. Nordstrom and Rundgren (2023) indicated that *L. rubellus* is less confined to soils with a certain pH and texture range than *L. terrestris*. This finding corresponds with our observation that considerable abundances of *L. rubellus* were found in loam poor and light loamy sand as well as loamy sand, while for *L. terrestris* they were especially found in the latter. Despite slight differences in habitat preference, it is very common for *L. rubellus* and *L. terrestris* to co-occur in European grasslands. We think it is likely that competition for food plays a role in the observed negative correlation between *L. terrestris* and *L. rubellus*.

4.5 Grassland age

Grassland age did not significantly correlate with *L. terrestris* population densities. This could be due to a relatively low number of young grasslands (≤ 3 years) in our dataset (n = 31), 11 young grasslands versus 20 old grasslands. We expected to find a positive correlation between the number of years without soil disturbance and the abundance of *L. terrestris*, as the negative influence of soil disturbance on earthworm population densities is broadly accepted (Decaëns et al., 2003; Lee, 1985; Lees et al., 2016; Low, 2016; Nieminen et al., 2011; Van Eekeren et al., 2008; Watkin & Wheeler, 1996). After disturbance by mechanical soil loosening, earthworm population density was found to recover within three years (Lees et al., 2016; Van Eekeren et al., 2008). Only Lees et al. (2016) specifically looked at anecics, which they found to recover three years after deep mechanical soil loosening.

Van Eekeren et al. (2008) argued that recovery of earthworm population density after disturbance is not only a function of time but also of the quality and amount of organic matter input (e.g. plant litter, manure) and the presence of an insulating vegetation

layer. Also, the presence of low-disturbance, resource-rich field margins hosting *L. terrestris* could improve the recovery of populations in the field after disturbance (Frazão et al., 2019; Nuutinen et al., 2011). Lower numbers of *L. terrestris* found in some young grasslands in our study could have been a combined result of recent soil disturbance, low organic matter input from the previous crop (maize) and, in some cases, a large distance from undisturbed field margins. Future research could further disentangle the effects of the pre-crop, management and semi-natural landscape elements in temperate grassland landscapes on anecic earthworm population density.

5. Conclusions

In this earthworm inventory in grasslands on sandy soil, we found that *L. terrestris* was more abundant in soils with a higher silt percentage, likely because of positive relationships between loaminess and other soil factors like soil moisture and nutrient retention, resulting in favourable living conditions. Unexpectedly, *L. terrestris* was also present in a grassland on loam-poor sand. Demonstrating the ability of the species to establish itself in sandy soil. The classification tree by Lindahl et al. (2009) correctly predicted the level of *L. terrestris* abundance based on land use and soil texture in 63% percent of our data points. A weak positive correlation was observed between *L. terrestris* population densities and gley depth. Gley depth is an indicator of maximum groundwater levels. Higher groundwater levels or soil profiles with temporarily waterlogged layers could create less hospitable, oxygen-poor environments for the deep-burrowing *L. terrestris*. Additionally, a negative correlation with epigeic earthworm *L. rubellus* abundance was shown. We suspect this is caused by competition for soil surface food resources, perhaps combined with slightly diverging soil habitat preferences. Overall, this correlative study provides further insights into *L. terrestris* habitat selection, which helps us understand the species' potential for water regulation in the widespread grassland agro-ecosystems.

6. Statements & declarations

This work was supported by the Public-Private Partnership KLIMAP. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Authors Roos van de Logt and Thom van der Sluijs contributed equally to the creation of the manuscript. Experimental design, data collection, writing and revision were done by

Roos van de Logt and Thom van der Sluijs. Data analysis was performed by Thom van der Sluijs. Experimental design, writing and revision of the manuscript were done by Nick van Eekeren. All authors read and approved of the final manuscript. The datasets generated during and/or analysed during the current study are available from the corresponding author on request.

7. Acknowledgements

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8. Supplementary materials

Supplementary Table S2.1 Linear models for describing the correlation between soil textural features and *Lumbricus terrestris* adult, juvenile and total population densities. log = logarithm transformed data; sqrt = square root transformed data; TotLT = Total *L. terrestris* density (m^{-2}); TotAdLT = Total *L. terrestris* adult density; TotJuvLT = Total *L. terrestris* juvenile density. %...10/40: soil silt or clay concentration at 0-10 or at 30-40 cm depth; Significance levels: $^{\#} < 0.05$, $^{\#\#} < 0.025$, $^{\#\#\#} < 0.001$.

Model description	R^2_{adj}	RMSE
log(TotLT+1) = -0.053 + 0.095*%Silt40$^{\#\#\#}$	0.21	1.86
log(TotAdLT+1) = 0.0055 + 0.060*%Silt40$^{\#\#\#}$	0.33	1.71
log(TotJuvLT+1) = -0.023 + 0.072*%Silt40$^{\#\#}$	0.15	1.40
log(TotLT+1) = 0.18 + 0.16*%Silt40$^{\#\#\#}$ – 0.56*%Clay10$^{\#\#}$	0.20	1.35
log(TotAdLT+1) = 0.12 + 0.092*%Silt40$^{\#\#\#}$ – 0.27*%Clay10$^{\#}$	0.11	1.98
log(TotJuvLT+1) = 0.20 + 0.14*%Silt40$^{\#\#\#}$ – 0.53*%Clay10$^{\#\#}$	0.21	1.85

Supplementary Table S2.2 Linear models for describing the correlation of gley depth and soil silt concentration with *Lumbricus terrestris* adult, juvenile and total population densities. log = logarithm transformed data; sqrt = square root transformed data; TotLT = Total *L. terrestris* density m^{-2}; TotAdLT = Total *L. terrestris* adult density m^{-2}; TotJuvLT = Total *L. terrestris* juvenile density m^{-2}. %silt10/40: soil silt concentration at 30-40 cm depth; StartGley = gley depth in centimeters. Significance level: $^{!} < 0.1$, $^{\#} < 0.05$, $^{\#\#} < 0.025$, $^{\#\#\#} < 0.001$.

Model description	R^2_{adj}	RMSE
log(TotLT+1) = -1.58$^{!}$ + 0.11*%Silt40$^{\#\#\#}$ + 0.016*StartGley$^{\#}$	0.25	1.80
sqrtTotAdLT = -1.83 + 0.12*%Silt40$^{\#\#\#}$ + 0.018*StartGley$^{!}$	0.19	2.21
log(TotJuvLT+1) = -1.39 + 0.089*%Silt40$^{\#\#}$ + 0.014*StartGley$^{!}$	0.14	1.93

Supplementary Table S2.3 Linear models for describing the correlation between *Lumbricus rubellus* population densities and *Lumbricus terrestris* adult, juvenile and overall population densities. log = logarithm transformed data; sqrt = square root transformed data; TotLT = Total *L. terrestris* density m^{-2}; TotAdLT = Total *L. terrestris* adult density; TotJuvLT = Total *L. terrestris* juvenile density; TotEnd = Total endogeics, TotLR = Total *L. rubellus*; %silt10/40: soil silt concentration at 30-40 cm depth; StartGley = gley depth in cm. Significance levels: $^{\#} < 0.05$, $^{\#\#} < 0.025$, $^{\#\#\#} < 0.001$.

Model description	R^2_{adj}	RMSE
$log(TotLT+1) = 2.93^{\#\#\#} - 0.0098*sqrtTotLR^{\#\#}$	0.10	1.99
$log(TotLT+1) = -0.66 + 0.10*\%Silt40^{\#\#\#} + 0.016*StartGley^{\#} - 0.11*sqrtTotLR^{\#}$	0.31	1.71
$sqrtTotAdLT = 3.27^{\#\#\#} - 0.17*sqrtTotLR^{\#\#}$	0.11	2.33
$log(TotAdLT+1) = -0.38 + 0.064*\%Silt40^{\#\#\#} + 0.011*StartGley^{\#} - 0.078*sqrtTotLR^{\#}$	0.24	1.30

Supplementary Table S2.4 Linear models for describing the correlation vertical burrow density, assumed to be (formerly) inhabited by *Lumbricus terrestris* individuals, as a function of soil textural features, gley depth and *Lumbricus rubellus* population density. sqrt = square root transformed data; Burrows = number of vertical burrows m^{-2}; %[Silt/Clay][10/40]: soil silt or clay concentration at 0-10 or at 30-40 cm depth (%); TotLR = total *L. rubellus* individuals m^{-2}; StartGley = gley depth in cm. Significance levels: $^{\#} < 0.05$, $^{\#\#} < 0.025$.

Model description	R^2_{adj}	RMSE
$sqrtBurrows = 3.06^{\#} + 0.10*\%Silt40^{\#}$	0.06	3.65
$sqrtBurrows = 3.29^{\#\#} + 0.17*\%Silt40^{\#\#} - 0.54*\%Clay10$	0.09	3.58
$sqrtBurrows = 7.23^{\#\#\#} - 0.28*sqrtTotLR^{\#\#}$	0.13	3.51
$sqrtBurrows = 1.44 + 0.12*\%Silt40^{\#\#} + 0.04*StartGley^{\#\#} - 0.25*sqrtTotLR^{\#\#}$	0.26	3.19
$sqrtBurrows = -0.68 + 0.15*\%Silt40^{\#\#} + 0.038*StartGley^{\#\#}$	0.15	3.44

Supplementary Table S2.5A Linear regression directions. %Lutum/Silt/Sand10/40 = percentage of silt/sand/lutum at 10/40 cm depth. Age = years since last grassland disturbance. AHorDepth = depth of the A-horizon. StartGley = depth at which gley was observed. pH/SOM10/40 = pH/Soil Organic Matter at 10/40 cm depth. TotAdLT = number of adult *Lumbricus terrestris*. TotJuvLT = number of juvenile *L. terrestris*. TotLT = total number of *L. terrestris*. AdLR = number of adult *Lumbricus rubellus*. JuvLR = number of juvenile *L. rubellus*. TotLR = total number of *L. rubellus*.

	%Lutum10	%Silt10	%Sand10	%Lutum40	%Silt40	%Sand40	Age	AHorDept	StartGley	pH10	SOM10	pH40	SOM40	TotAdLT	TotJuvLT	TotLT	AdLR	JuvLR	TotLR
%Lutum10	1	0.66	-0.75	0.93	0.68	-0.76	0.05	-0.17	-0.62	-0.03	0.14	0.28	0.08	0.1	-0.02	0	-0.18	-0.01	-0.1
%Silt10	0.66	1	-0.99	0.59	0.97	-0.96	-0.14	-0.16	-0.4	-0.07	0.03	0	0.25	0.36	0.26	0.29	-0.33	-0.15	-0.27
%Sand10	-0.75	-0.99	1	-0.68	-0.97	0.97	0.12	0.17	0.45	0.07	-0.05	-0.05	-0.23	-0.33	-0.23	-0.26	0.32	0.13	0.25
%Lutum40	0.93	0.59	-0.68	1	0.65	-0.75	0.01	-0.09	-0.58	-0.01	0.11	0.24	0.06	0.09	-0.04	-0.02	-0.23	0.04	-0.09
%Silt40	0.68	0.97	-0.97	0.65	1	-0.99	-0.17	-0.11	-0.37	-0.11	0	-0.04	0.24	0.36	0.28	0.31	-0.34	-0.11	-0.25
%Sand40	-0.76	-0.96	0.97	-0.75	-0.99	1	0.14	0.11	0.43	0.1	-0.02	-0.01	-0.22	-0.33	-0.24	-0.27	0.34	0.09	0.24
Age	0.05	-0.14	0.12	0.01	-0.17	0.14	1	-0.06	-0.06	0.36	0.78	0.5	0.27	-0.2	-0.13	-0.16	0.19	0.38	0.35
AHorDepth	-0.17	-0.16	0.17	-0.09	-0.11	0.11	-0.06	1	0.42	0.12	-0.19	-0.17	-0.05	-0.09	-0.12	-0.12	-0.2	0.17	0.02
StartGley	-0.62	-0.4	0.45	-0.58	-0.37	0.43	-0.06	0.42	1	-0.02	-0.15	-0.34	0.05	0.07	0.03	0.04	0.09	0.18	0.17
pH10	-0.03	-0.07	0.07	-0.01	-0.11	0.1	0.36	0.12	-0.02	1	0.56	0.71	0.47	-0.02	0.05	0.04	-0.02	0.22	0.14
SOM10	0.14	0.03	-0.05	0.11	0	-0.02	0.78	-0.19	-0.15	0.56	1	0.68	0.62	-0.09	-0.11	-0.12	0.15	0.26	0.25
pH40	0.28	0	-0.05	0.24	-0.04	-0.01	0.5	-0.17	-0.34	0.71	0.68	1	0.31	-0.04	0	-0.01	0.13	0.17	0.18
SOM40	0.08	0.25	-0.23	0.06	0.24	-0.22	0.27	-0.05	0.05	0.47	0.62	0.31	1	0.19	0.13	0.15	-0.04	0.13	0.07
TotAdLT	0.1	0.36	-0.33	0.09	0.36	-0.33	-0.2	-0.09	0.07	-0.02	-0.09	-0.04	0.19	1	0.51	0.64	-0.3	-0.27	-0.33
TotJuvLT	-0.02	0.26	-0.23	-0.04	0.28	-0.24	-0.13	-0.12	0.03	0.05	-0.11	0	0.13	0.51	1	0.99	-0.23	-0.13	-0.2
TotLT	0	0.29	-0.26	-0.02	0.31	-0.27	-0.16	-0.12	0.04	0.04	-0.12	-0.01	0.15	0.64	0.99	1	-0.26	-0.16	-0.24
AdLR	-0.18	-0.33	0.32	-0.23	-0.34	0.34	0.19	-0.2	0.09	-0.02	0.15	0.13	-0.04	-0.3	-0.23	-0.26	1	0.4	0.78
JuvLR	-0.01	-0.15	0.13	0.04	-0.11	0.09	0.38	0.17	0.18	0.22	0.26	0.17	0.13	-0.27	-0.13	-0.16	0.4	1	0.89
TotLR	-0.1	-0.27	0.25	-0.09	-0.25	0.24	0.35	0.02	0.17	0.14	0.25	0.18	0.07	-0.33	-0.2	-0.24	0.78	0.89	1

Supplementary Table S2.5B Significance of Pearson's r correlation coefficient

	%Lutum10	%Silt10	%Sand10	%Lutum40	%Silt40	%Sand40	Age	AHorDepth	StartGley	pH10	SOM10	pH40	SOM40	TotAdLT	TotJuvLT	TotLT	AdLR	JuvLR	TotLR
%Lutum10		**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.7163	0.1897	**0.000**	0.8438	0.2653	0.0264	0.5262	0.4544	0.8695	0.9875	0.1623	0.9249	0.4443
%Silt10	**0.000**		**0.000**	**0.000**	**0.000**	**0.000**	0.2615	0.2262	0.0013	0.5947	0.8172	0.9924	0.0511	0.0046	0.0426	0.0201	0.0087	0.2468	0.0346
%Sand10	**0.000**	**0.000**		**0.000**	**0.000**	**0.000**	0.3492	0.1992	0.0002	0.6146	0.7024	0.7226	0.0673	0.0087	0.0778	0.0404	0.0108	0.2979	0.0462
%Lutum40	**0.000**	**0.000**	**0.000**		**0.000**	**0.000**	0.909	0.4827	**0.000**	0.9438	0.3784	0.057	0.6438	0.4955	0.7719	0.8886	0.0757	0.7648	0.4967
%Silt40	**0.000**	**0.000**	**0.000**	**0.000**		**0.000**	0.1998	0.4073	0.0033	0.4075	0.9832	0.7357	0.0619	0.0042	0.028	0.0129	0.0061	0.3796	0.0486
%Sand40	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**		0.272	0.3945	0.0006	0.4612	0.8897	0.9663	0.087	0.009	0.0633	0.0329	0.0065	0.4756	0.0651
Age	0.7163	0.2615	0.3492	0.909	0.1998	0.272		0.6554	0.6415	0.004	**0.000**	**0.000**	0.0334	0.1212	0.3003	0.2285	0.1367	0.0025	0.0047
AHorDepth	0.1897	0.2262	0.1992	0.4827	0.4073	0.3945	0.6554		0.0007	0.3343	0.1468	0.1896	0.6727	0.4859	0.3623	0.3465	0.1267	0.1798	0.8806
StartGley	**0.000**	0.0013	0.0002	**0.000**	0.0033	0.0006	0.6415	0.0007		0.859	0.2414	0.0075	0.7033	0.5761	0.8335	0.7738	0.4839	0.1677	0.1939
pH10	0.8438	0.5947	0.6146	0.9438	0.4075	0.4612	0.004	0.3343	0.859		**0.000**	**0.000**	0.0001	0.8847	0.6954	0.7445	0.8563	0.091	0.29
SOM10	0.2653	0.8172	0.7024	0.3784	0.9832	0.8897	**0.000**	0.1468	0.2414	**0.000**		**0.000**	**0.000**	0.502	0.3827	0.3666	0.2522	0.0433	0.0492
pH40	0.0264	0.9924	0.7226	0.057	0.7357	0.9663	**0.000**	0.1896	0.0075	**0.000**	**0.000**		0.0158	0.7464	0.9982	0.9561	0.3144	0.1864	0.1569
SOM40	0.5262	0.0511	0.0673	0.6438	0.0619	0.087	0.0334	0.6727	0.7033	0.0001	**0.000**	0.0158		0.15	0.3159	0.248	0.7307	0.3016	0.5948
TotAdLT	0.4544	0.0046	0.0087	0.4955	0.0042	0.009	0.1212	0.4859	0.5761	0.8847	0.502	0.7464	0.15		**0.000**	**0.000**	0.0191	0.0342	0.0079
TotJuvLT	0.8695	0.0426	0.0778	0.7719	0.028	0.0633	0.3003	0.3623	0.8335	0.6954	0.3827	0.9982	0.3159	**0.000**		**0.000**	0.0761	0.3281	0.1174
TotLT	0.9875	0.0201	0.0404	0.8886	0.0129	0.0329	0.2285	0.3465	0.7738	0.7445	0.3666	0.9561	0.248	**0.000**	**0.000**		0.0444	0.2113	0.0609
AdLR	0.1623	0.0087	0.0108	0.0757	0.0061	0.0065	0.1367	0.1267	0.4839	0.8563	0.2522	0.3144	0.7307	0.0191	0.0761	0.0444		0.0013	**0.000**
JuvLR	0.9249	0.2468	0.2979	0.7648	0.3796	0.4756	0.0025	0.1798	0.1677	0.091	0.0433	0.1864	0.3016	0.0342	0.3281	0.2113	0.0013		**0.000**
TotLR	0.4443	0.0346	0.0462	0.4967	0.0486	0.0651	0.0047	0.8806	0.1939	0.29	0.0492	0.1569	0.5948	0.0079	0.1174	0.0609	**0.000**	**0.000**	



Chapter 3

Earthworm communities are more affected by tillage than soil cover loss in an on-farm mesocosm-study in grassland

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Abstract

Earthworms are ecosystem engineers that support agricultural grasslands in providing ecosystem services such as water regulation, nutrient cycling and soil quality. However, the effects of grassland renewal interventions such as tillage and removal of vegetation cover on indigenous earthworm communities are poorly understood. In particular, species-specific responses, demographic patterns and the influence of weather conditions are understudied. In an on-farm mesocosm experiment, we tested the effects of four treatments, combinations of tillage versus no-tillage and bare versus covered soil, on indigenous earthworm communities six months after treatment. The experiment followed a randomised block design with five replicates and was conducted over two consecutive years, with the first year being drier than the second. Both tillage and soil bareness decreased earthworm abundance and biomass, resulting in 330 ind. and 131 g m^{-2} in undisturbed grassland versus 81 ind. and 36 g m^{-2} in tilled bare soil. Demographic structure and species composition were unaffected. Endogeic earthworms were the most abundant ecological group (79%) and were most strongly affected by the treatments. Epigeic and anecic earthworms showed an interaction effect between treatment and year, suggesting that their responses to tillage and soil bareness were influenced by weather conditions. These outcomes demonstrate that different grassland renewal strategies can affect ecological groups of earthworms in distinct ways. Moreover, the interplay between management strategies and weather conditions appears to be important. Further long-term field studies in open plots are needed to refine management strategies that support earthworm communities.

Keywords: Earthworms; grassland; mesocosm; epigeic; endogeic; anecic; ley

Highlights

- Abundance and biomass were highest in permanent grassland and lowest in tilled bare soil
- Tillage had a stronger negative effect on the earthworm community than soil cover loss
- Demography was affected by weather, but not by tillage and vegetation cover
- After six months, Shannon-index was neither affected by tillage nor vegetation cover

1. Introduction

Agricultural grasslands provide a wide range of provisional, regulating and cultural ecosystem services (Zhao et al., 2020; Schils et al., 2022). Grassland associated soil biota, such as earthworms, are key contributors to these services. They play a crucial role in water regulation (Ehlers, 1975; Clements et al., 1991; Valckx et al., 2009), nutrient cycling (Curry & Byrne, 1992; Whalen & Parmelee, 2000; Blouin et al., 2013) and soil fertility (Syers & Springett, 1984; Bhadauria & Saxena, 2010). In temperate regions, grasslands can be exceptionally rich in earthworms. They are generally inhabited by 300-500 earthworms m^{-2} (Cole et al., 2006; Rutgers et al., 2016; Hoeffner, Santonja et al., 2021), with some exceptions reaching > 1,000 earthworms m^{-2} (Rutgers et al., 2016). As ecosystem engineers, earthworms affect the biological, chemical and physical properties of their environment (Lavelle et al., 1997). In turn, farmers' soil management practices directly and indirectly affect the earthworm community (Lofs-Holmin, 1983; Kladivko & Timmenga, 1990; Hoeffner, Santonja et al., 2021). For example in the case of grassland renewal, which is an important management tool to improve the botanical composition of intensively used grasslands (Andueza et al., 2016). However, the interaction between agricultural management practices and the earthworm community is not always taken into account in decision making about grassland renewal strategies, neither on-farm, nor in the design of grassland renewal legislation.

During grassland renewal, the old sward is usually destroyed by either herbicide application or tillage (rotavating and ploughing) or a combination of both. Two common reseeding strategies are either direct reseeding, or one season of arable cropping before reseeding the grassland. In both cases, renewal causes a disruption of the steady supply of food and insulation that permanent grassland offers to earthworms. Especially the second strategy severely decreases the input of plant material that earthworms can feed on for a prolonged period of time, because annual arable crops provide less litter than grass does. Conversion from grassland to arable land is known to cause a decline in earthworm abundance and species diversity (Van Eekeren et al., 2008). Furthermore, tillage can have a negative impact on earthworm abundance (Chan, 2001; Briones & Schmidt, 2017; Lori et al., 2025). This is due to mechanical damage to earthworms (Gerard & Hay, 1979) or their burrows, exposure to predators (Briones & Schmidt, 2017) and redistribution of food sources (Curry & Schmidt, 2007). Not all earthworm species respond equally to tillage; this depends on their functional traits and ecological niche (Wyss & Glasstetter, 1992; Ivask et al., 2007; Capowiez, Cadoux, Bouchant et al., 2009). Earthworms are often divided into the following three

ecological categories: Epigeic earthworms mainly live and feed in the top soil and litter layer, endogeic earthworms live and feed in the first 20-40 cm of the soil and anecic earthworms make vertical burrows (up to 2 m deep) and feed on leaf litter which they drag into their burrows. Some studies reported that epigeics are insensitive to tillage (Van Capelle et al., 2012; Pelosi et al., 2014), while others reported negative effects (Nuutinen, 1992; Van Eekeren et al., 2008; Briones & Schmidt, 2017; Sleiderink et al., 2024). Epigeic earthworms are usually abundant in grasslands, but not in arable systems, due to absence of a litter layer (Van Eekeren et al., 2008; Józefowska et al., 2016). Endogeics show variable responses to tillage regimes, ranging from negative (De Oliveira et al., 2012) to neutral (Peigné et al., 2009) and positive responses (Wyss & Glasstetter, 1992; Pelosi et al., 2009). In arable soils, endogeics make up the largest part of the earthworm community, as they do not need the litter layer to feed on (Van Eekeren et al., 2008; Józefowska et al., 2016; Mazur-Pączka et al., 2019). Anecic species, particularly *Lumbricus terrestris*[1], have consistently been found to decline under tillage (Nuutinen, 1992; Capowiez, Cadoux, Bouchant et al., 2009; Peigné et al., 2009). Anecic earthworms are scarcely found in arable soils, as they depend on the litter layer for food (Prendergast-Miller et al., 2021; Hoeffner et al., 2022).

There are several knowledge gaps concerning earthworm community dynamics in response to tillage and soil cover loss associated with grassland renewal. First, most studies on the effect of tillage on earthworms have been conducted in arable systems, probably because tillage is more commonly associated with arable cropping. However, earthworm communities in grassland are generally distinct from those in arable land (Mazur-Pączka et al., 2019; Hoeffner, Hotte et al., 2021). Therefore, grassland-specific research is required. Second, studies that include information about juveniles at the species level are exceedingly rare (Evans & Guild, 1948; Briones & Schmidt, 2017), even though juveniles sometimes constitute over 60% of the total population (Singh et al., 2021; Van de Logt et al., 2023b), making demography a factor to be considered. Third, detailed information about specific tillage practices and weather conditions often remains undocumented (Chan, 2001), while the effects of tillage can depend on the type of tillage applied (Emmerling, 2001; Capowiez, Cadoux, Bouchant et al., 2009), and weather conditions can have a strong and immediate effect on earthworm communities (Singh et al., 2019). Given that earthworms play a

[1] *L. terrestris* is considered epi-anecic (Hoeffner et al., 2018; Bottinelli et al., 2020), but to avoid overlong terminology, we will use 'anecic' for both epi-anecic and anecic species (in this paper, *L. terrestris* and *A. nocturna*, respectively).

crucial role in grassland functioning, the effects of tillage and soil cover loss that are associated with grassland renewal should not be overlooked. Therefore, we argue that field experiments that address the species level, demography, weather conditions and tillage specifications are crucial to expanding our understanding of earthworm population dynamics under different grassland renewal strategies.

To investigate the effect of two grassland renewal-associated management practices (tillage and temporary soil cover loss) on earthworm communities, we conducted an on-farm experiment for two consecutive years. As it can be challenging to isolate the factors that influence earthworm populations in the field, we used mesocosms. Mesocosms provide a semi-controlled environment that can help to bridge the gap between laboratory studies and open field experiments (Lubbers & Groenigen, 2013; Andriuzzi et al., 2015). The four grassland renewal strategies that were simulated in the mesocosms were permanent grassland (i.e. no renewal), grassland renewal with tillage, and temporary conversion to arable land with and without tillage. We hypothesised that both tillage and soil bareness would negatively affect earthworm abundance, total biomass and species diversity for adults as well as juveniles. We also expected ecological groups to respond differently, with epigeic and anecic species showing the strongest negative responses. The experimental treatments and their corresponding hypotheses are summarised in Table 3.1.

Table 3.1 Overview of the management practices that were simulated, the treatment names, and the hypothesised effects on earthworm abundance and species diversity. The green box (top left) contains the consequences of the treatments that are expected to be beneficial to earthworm abundance and diversity, the red box (bottom right) contains the consequences of the treatments that are expected to be detrimental to earthworm abundance and diversity, and the two yellow boxes contain the treatments that are expected to have an intermediate effect (neither beneficial nor detrimental) on earthworm abundance and diversity.

Soil cover / Tillage	Present	Absent
No	**Simulated management practice:** continuation of permanent grassland **Treatment:** NoTillGrass **Hypothesis:** high abundance, high diversity	**Simulated management practice:** conversion of permanent grassland to arable land, without tillage **Treatment:** NoTillBare **Hypothesis:** intermediate abundance, intermediate diversity
Yes	**Simulated management practice:** renewal of permanent grassland, with tillage **Treatment:** TillGrass **Hypothesis:** intermediate abundance, intermediate diversity	**Simulated management practice:** conversion of permanent grassland to arable land, with tillage **Treatment:** TillBare **Hypothesis:** low abundance, low diversity

2. Materials and methods

2.1 Study area

The field experiment was conducted in 2011-2012 on a seven-year-old grass-clover pasture on sandy soil at an organic dairy goat farm in Biezenmortel, the Netherlands (51°37'50.6"N 5°11'25.3"E). Before setting up the field experiment, baseline measurements were conducted to assess initial soil properties and earthworm community (Table 3.2). Soil samples and soil cores were collected from the 3x30 m plot in which the experiment was to be installed. A soil gouge auger was used to take a representative number of soil samples to a depth of 10 cm; the samples were distributed evenly over the 3x30 m plot. Samples were pooled and mixed thoroughly before chemical analysis by Eurofins, Wageningen. The organic matter content of the soil was 4.9%, pH-KCl was 5.6 and total N was 1.95 g per kg dry soil. The earthworm community was assessed by hand-sorting six soil cores (20 x 20 x 20 cm) and collecting all the earthworms that they contained. Subsequently, 0.01% AITC solution (prepared by dissolving 1 mL allyl-isothiocyanate in 200 mL isopropanol and diluting it with 10 L water; Zaborski, 2003) was applied to the bottom of the pit to drive out and collect earthworms from the deeper soil layers. AITC solution was poured onto the soil surface until it did not infiltrate the soil anymore. Emerging earthworms were collected and rinsed with tap water. This procedure was continued until no more earthworms surfaced and at least 20-30 minutes had passed. All earthworms were stored in 70% ethanol for later species determination. Mean earthworm abundance was 184 ind. m^{-2}, and all three ecological groups of earthworms (epigeic, endogeic and anecic) were represented (Table 3.2).

Compared to the long-term average of the Netherlands, the spring of 2011 was very dry, followed by a very wet summer, and the autumn of 2011 was dry again. In 2012, spring was relatively wet, summer was wet, but less so than 2011, and the autumn was again dry (Table 3.3).

Table 3.2 Results of baseline assessment of the experimental site

Baseline assessment	
Total earthworm community	
Mean total abundance (ind. m^{-2})	184
Mean total biomass (ind. m^{-2})	80
Juvenile fraction	0.66
Adult earthworm community	
Epigeics (%)	46
Endogeics (%)	39
Anecics (%)	15
Soil properties	
pH-KCl	5.6
Organic matter (%)	4.9
N-total (g N/kg dry soil)	1.95
P-Al (mg P_2O_5 100 g dry $soil^{-1}$)	65
Clay (%)	2
Silt (%)	16
Sand (%)	77

Table 3.3 Overview of rainfall data (mm) of the Netherlands in 2011 and 2012 as compared to the Dutch long-term average. Relatively dry months are marked in red, and relatively wet months are marked in green.

		Cumulative rainfall		**Difference**	
Season	**Long-term average**	**2011**	**2012**	**2011**	**2012**
Spring	172	49	138	-123	-34
Summer	225	350	286	125	61
Autumn	243	142	215	-101	-28
Winter	210	244		34	

2.2 Installation of the mesocosm field experiment

In May 2011, a mesocosm field experiment was established in a randomised block design with four treatments (Figure 3.1) and five replicates per treatment, constituting a total of twenty mesocosms. The total surface area of the field experiment was 3 m in width and 30 m in length. Twenty 1 m^2 plots were allocated, and the soil in the plots was prepared for the various treatments (Table 3.1). In the TillGrass, TillBare and NoTillBare plots, the grass-clover sward was removed using a sod cutter. In both 'Till' treatments, this was followed by tillage to 20-25 cm depth using a rotavator, followed by a spading machine. For the NoTillGrass treatment, the plots were reseeded with grass-clover (*Lolium perenne* and *Trifolium repens*, respectively). The NoTillGrass plots received no treatment. After preparation of the soil, the mesocosms were installed,

each of which were spaced 90 cm apart. Using a crane, steel pipes (ø 61 cm, height 50 cm) were driven 40 cm into the soil, leaving a 10 cm ring above the soil surface (Figure 3.1). The NoTillBare and TillBare plots were kept bare throughout the experiment by regular hand-weeding.

The mesocosms were covered with a 1.5 mm mesh gauze, which was fixed with a lashing strap both to prevent earthworms from escaping (Figure 3.1) and to protect them from predators. A fence was placed around the mesocosms to prevent grazing animals from entering the plot and disturbing the experiment. After the November 2011 harvest (Section 2.3), the mesocosms were removed from the soil. In May 2012, the mesocosms were re-installed, in the same grassland and adjacent to the 2011 plot. Installation was again carried out as described for the 2011 experiment.

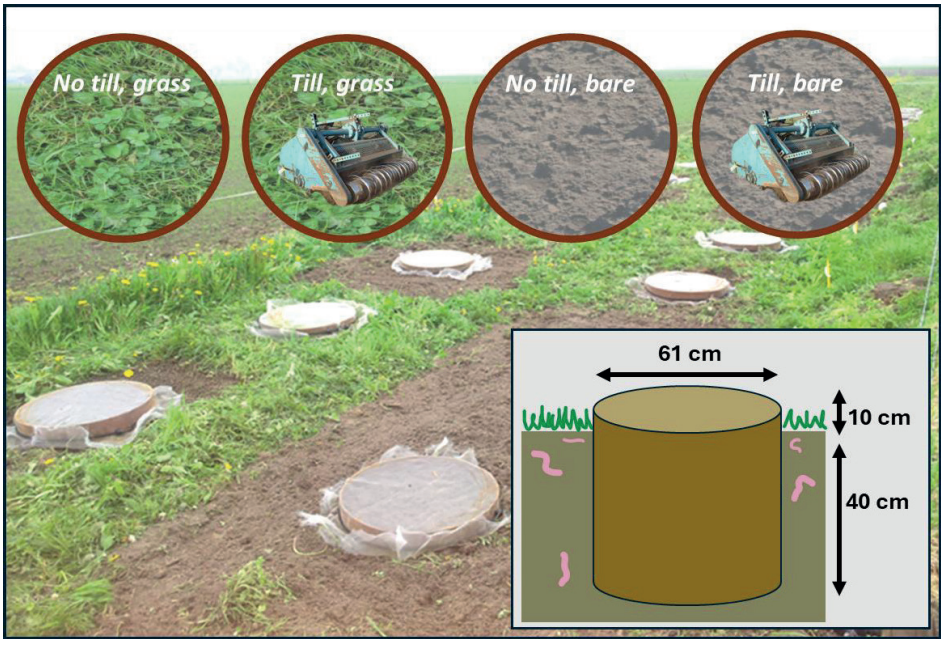

Figure 3.1 Photo of the mesocosm field-experiment (background), schematic overview of an installed mesocosm and its size dimensions (bottom-right corner), and visualisations of the four different treatments (upper half). The image used to indicate "till" treatments is a photo of the actual rotavator that was used in the experiment.

2.3 Harvesting of the field experiment

In both 2011 and 2012, the mesocosms were harvested in November, six months after installation. The harvesting procedure consisted of carefully removing the top 20 cm of the soil within the mesocosm using a shovel. The removed soil was placed

in a mortar tub and hand-sorted in the field to collect all earthworms present in this layer. Subsequently, earthworms from deeper soil layers were collected using mustard extraction with 0.01% AITC solution, following the same procedure as described in section 2.1. Total earthworm biomass per mesocosm was determined. Earthworm identification was carried out using Sims and Gerard's approach (1985). There were a small number of damaged earthworms that could not be identified; these were included in the total biomass but not in species determination.

2.4 Statistical analysis

Prior to analysis, the collected data was checked for normality and log or square root transformed when needed (transformation always resulted in normal distribution). Rstudio (version 2023.12.0 + 369) was used to perform linear regression analyses and ANOVA. Packages used for analysis were car (Fox and Weisberg, 2019), sjplot (Lüdecke, 2024), ggplot2 (Wickham, 2016), lme4 (Batesbert et al., 2015), multcomp (Hothorn et al., 2008), multcompView (Graves et al., 2024), emmeans (Lenth, 2025) and dplyr (Wickham et al., 2023). Factors were *treatment* (NoTillGrass, NoTillBare, TillGrass and TillBare) and *year* (1 and 2).

3. Results

3.1 Total abundance and biomass

We found significant treatment and year effects on earthworm abundance and biomass (all $p < 0.001$, Table 3.4). There was no interaction between year and treatment for abundance and biomass of the total population. The highest earthworm abundance and biomass were found in NoTillGrass, with 330 ind. m^{-2}, amounting to a biomass of 131 g m^{-2}. TillGrass resulted in a significantly lower mean abundance and biomass, of 142 ind. m^{-2} and 62 g m^{-2}, respectively. NoTillBare yielded a mean abundance of 228 ind. m^{-2}, amounting to a biomass of 89 g m^{-2}, which was significantly higher than TillBare. TillBare resulted in the lowest earthworm abundance and biomass, with 81 ind. m^{-2} and 36 g m^{-2}, respectively. Individual biomass was not affected by the treatments. Total earthworm abundance and biomass were both higher in year 2 (281 ind. m^{-2} and 107 g m^{-2}) than in year 1 (110 ind. m^{-2} and 52 g m^{-2}). The individual biomass of earthworms was significantly higher in year 1 (0.64 g n^{-1}) than in year 2 (0.39 g $ind.^{-1}$).

Table 3.4 Abundance and biomass of earthworms over the treatments and years (main effects; interactions shown in Table 3.5). Values followed by the same letter (a-c) within a row are not statistically different at the 5% error level for the main effect. P-values: * $p < 0.05$, **$p < 0.01$, *** $p < 0.001$. NB: Total ≠ Adult + Juvenile or Epigeic + Endogeic + Anecic, as it also includes a small group of damaged earthworms that could not be assigned to a category.

		Treatment				Year				
Group	**Unit**	**No till, grass**	**No till, bare**	**Till, grass**	**Till, bare**	**1**	**2**	**Treat-ment (T) p-value**	**Year (Y) p-value**	**T*Y p-value**
Total	$n.m^{-2}$	330 a	228 ab	142 bc	81 c	110 a	281 b	***	***	ns
Biomass	$g.m^{-2}$	131 a	89 ab	62 bc	36 c	52 a	107 b	***	***	ns
Ind. biomass	$g.n^{-1}$	0.44	0.43	0.52	0.66	0.64 a	0.39 b	ns	**	ns
Adult	$n.m^{-2}$	125 a	79 b	46 c	23 d	45 a	92 b	***	***	ns
Juvenile	$n.m^{-2}$	192 a	131 ab	91 b	55 b	57 a	178 b	***	***	ns
Juvenile frac.		0.53	0.51	0.60	0.55	0.45 a	0.65 b	ns	***	ns
Epigeic	$n.m^{-2}$	41	26	22	11	20	30	***	*	*
Endogeic	$n.m^{-2}$	261 a	169 ab	100 bc	60 c	73 a	222 b	***	***	ns
Anecic	$n.m^{-2}$	16	16	16	7.1	9.0	18	*	***	*

3.2 Population demography

Adults showed a clear treatment effect, as they were most abundant in NoTillGrass, followed by NoTillBare, TillGrass and TillBare ($p < 0.001$, Table 3.4). Juvenile mean abundance followed a similar pattern, although the juvenile fraction was not significantly affected by the treatments. Both adults and juveniles were more abundant in year 2 than in year 1 ($p < 0.001$ in both cases). The juvenile fraction in year 1 (0.45) was lower than in year 2 (0.65, $p < 0.001$).

3.3 Ecological groups

Of the total number of earthworms found, 13% of the individuals were epigeics, 79% were endogeics and 7% were anecics. All three groups were present in both years and in all treatments. Endogeic earthworms represented the dominant ecological group in all treatments and both years (Table 3.4). This group was strongly affected by treatment and year (both main effects $p < 0.001$). Endogeics were most abundant in NoTillGrass, followed by NoTillBare, TillGrass, and lastly TillBare. In year 2, endogeic abundance had tripled compared to year 1 (222 versus 73 ind. m^{-2}). Epigeic and anecic abundance showed a different pattern in their response to the treatments than did endogeic abundance, with both epigeic and anecic abundance showing an interaction between treatment and year (Tables 3.4 and 3.5).

Table 3.5 Abundance of epigeic and anecic earthworms over the treatment x year interaction (significant interaction effect, see Table 3.4). Significance letters refer to means within one ecological group.

		Treatment			
		No till, grass	**No till, bare**	**Till, grass**	**Till, bare**
Epigeic	Year 1	40 a	26 ab	7.1 b	6.4 b
	Year 2	42 a	25 ab	36 a	16 ab
Anecic	Year 1	11 ab	5.0 b	13 ab	7.1 b
	Year 2	21 ab	27 a	18 ab	7.1 b

Epigeic species were less abundant than endogeic species but more abundant than anecic species (Table 3.4). Epigeic abundance was highest in NoTillGrass, intermediate in NoTillBare and lowest in TillBare (Tables 3.4 and 3.5). TillGrass showed a year effect: low numbers were found in year 1 and high numbers in year 2. Anecic species were present in low abundance, ranging from 0 to 35 ind. m^{-2} at the mesocosm level (data not shown). In both years, anecic abundance was intermediate in NoTillGrass

and TillGrass, and low in TillBare. NoTillBare showed a year effect: lower abundance was found in year 1 and higher abundance in year 2.

3.4 Species diversity

In total, eight earthworm species were identified during the experiment (Figure 3.2, Supplementary Table S3.1): *Aporrectodea caliginosa*, *Aporrectodea limicola*, *Aporrectodea rosea*, *Lumbricus rubellus*, *Lumbricus terrestris*, *Lumbricus castaneus*, *Octolasion lacteum* and *Aporrectodea nocturna* (ranked according to total abundance, from high to low). Not all species were found in both years; *O. lacteum* was only found in year 1 and *A. limicola* was only found in year 2. Shannon indices of species diversity showed no significant effects (Supplementary Table S3.1). At individual species level, some treatment effects were identified: *L. rubellus*, *A. rosea* and *Aporrectodea caliginosa* were more abundant in NoTillGrass than in TillBare. *L. castaneus* and *L. terrestris* showed a significant treatment × year interaction (Supplementary Table S3.1), which caused the treatment x year interaction effect in the groups of epigeics and anecics respectively. *O. lacteum* and *A. limicola* abundance showed no response to treatments.

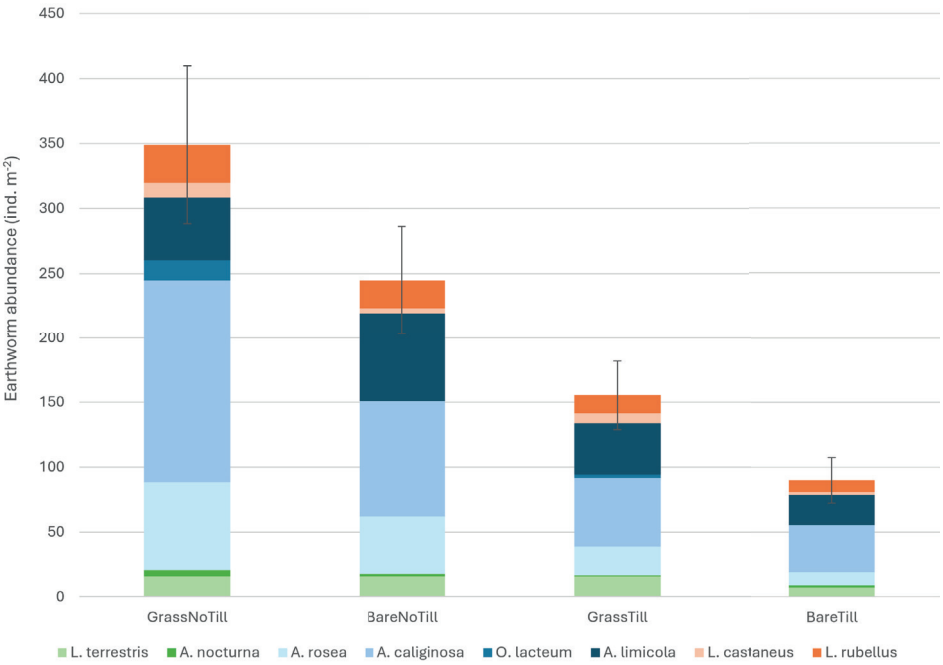

Figure 3.2 Cumulative abundance of earthworm species per treatment. Shades of green represent anecic species, shades of blue represent endogeic species, shades of orange represent epigeic species. Error bars and significance letters refer to the total earthworm abundance. The means per species and ANOVA outcomes are presented in Supplementary Table S3.1.

4. Discussion

4.1 Earthworm abundance and biomass is affected by soil cover, disturbance and weather

In line with our expectations, we found that total earthworm abundance and biomass were highest and lowest in the most strongly contrasting treatments, namely NoTill-Grass and TillBare, respectively (Table 3.4). Treatments had no effect on individual biomass. It has been demonstrated repeatedly that undisturbed grassland better supports earthworm communities than temporary grassland (Van Eekeren et al., 2008; Skuodienė et al., 2019; Van Eekeren, Dekker et al., 2025) or arable land (Springett, 1992; Boag et al., 1997; Didden, 2001), mainly because undisturbed grassland supplies a large and constant source of food and shelter, without any direct physical damage to the earthworms and no disturbance of the earthworms' living environment (Didden, 2001; Birkás et al., 2004; Curry, 2004).

In contrast to previous research, the set-up of this field mesocosm-experiment provides indications of the distinct roles that tillage and soil cover loss can play in shaping the earthworm community. The four treatments that were applied may each be considered a combination of two aspects that are either positive or negative for earthworms, namely disturbance: tillage (-) or no-tillage (+), and soil cover: grass (+) or bare soil (-) (Table 3.1). As expected, NoTillGrass (+ +) and TillBare (- -) result in the highest and lowest abundance and biomass, respectively.

In the intermediate treatments, a combination of one positive and one negative component was implemented: NoTillBare (+ -) and TillGrass (- +). The earthworm abundance and biomass under NoTillBare was close to the results in NoTillGrass, whereas the earthworm abundance and biomass in the TillGrass treatment were more similar to TillBare. Thus, the absence of tillage seems more important in maintaining the earthworm community than the presence of grass. This finding aligns with observations from Eriksen-Hamel et al. (2009), who showed in a long-term experiment on temperate grassland on sandy loam that conventional tillage decreased earthworm abundance, compared to no-tillage, irrespective of crop residue management. The authors proposed that soil disturbance is a more important driver than food availability.

Tillage can affect earthworms in several ways, it can cause physical harm and it redistributes organic matter through the soil. Feeding is more difficult for earthworms

when soil organic matter is no longer concentrated in the first few centimetres under the surface (Curry & Schmidt, 2007). In conclusion, both tillage and soil bareness decrease earthworm abundance and biomass in grassland systems, but tillage shows a more severe effect than soil bareness. This was observed in two consecutive years, of which the first was relatively dry and the second relatively wet.

4.2 Earthworm demography is affected by soil cover, disturbance and weather

In accordance with our expectations, we observed a similar effect on adult and juvenile abundance, six months after application of the treatments (NoTillGrass > NoTillBare > TillGrass > TillBare, Table 3.4). However, the effect was stronger among adults, where each treatment yielded a significantly different abundance. Among juveniles, the differences between the treatments were smaller (Table 3.4). This finding challenges the hypothesis by Bertrand et al. (2015) and Pelosi et al. (2017), that juveniles are more susceptible to mechanical damage and to low food availability than adults. Furthermore, our results do not align with a number of studies that reported tillage to be more detrimental to juveniles than adults (St. Remy & Day-Nard, 1982; Emmerling, 2001; Ulrich et al., 2010; Kuntz et al., 2013; Giannitsopoulos et al., 2020). We propose that juveniles are not necessarily more susceptible to mechanical damage, as small-bodied earthworms have a smaller chance of being harmed during mechanical disturbance (Wyss & Glasstetter, 1992). In addition, Lee (1985) and Kuntz et al. (2013) suggest that after tillage, earthworms allocate more energy to the reconstruction of burrows and less to reproduction, resulting in lower juvenile abundance.

Adults and juveniles were more abundant in the experiment of year 2 than in the experiment of year 1. There was a year-effect on the size of the juvenile fraction, which was 45% in year 1 and 65% in year 2. This was also reflected in the individual biomass, which was highest in year 1 (0.64 g ind.$^{-1}$ vs 0.39 g ind.$^{-1}$). This is probably related to weather conditions, as the spring of year 1 had a greater precipitation deficit than that of year 2 (-123 mm and -34 mm resp., when compared to the long-term average (Table 3.3). Adequate moisture conditions are crucial for earthworm abundance (Curry, 2004; Ivask et al., 2007) and reproduction (Singh et al., 2019). The dry spring and autumn of year 1 may have caused cocoon dehydration, which can retard embryonic development (Holmstrup, 1994). Additionally, the overall lower density of adult earthworms in year 1 could also have resulted in less interaction between earthworms compatible for mating, leading to a smaller juvenile fraction as a consequence.

4.3 Ecological groups respond differently to tillage and soil bareness

The hypothesis that epigeic earthworm abundance would be negatively affected by the tillage and bare soil was not fully supported (Table 3.1). We found an interaction between treatment and year for epigeics (Table 3.4 and 3.5). In TillGrass, epigeics were more abundant in year 2 than in year 1. The negative effect of tillage may have been overruled by the more favourable weather conditions in year 2 and the quick recovery of epigeic earthworms. In the TillBare treatment, we observed a similar trend (although non-significant). We were surprised not to find a negative effect of tillage on epigeic earthworms in both years, as a meta-analysis by Briones and Schmidt (2017) showed that the abundance of epigeic earthworms generally decreases under tillage. A smaller proportion of epigeics in more intensive tillage systems was found in an analysis of compiled data from studies conducted over a period of 21 years (Sutri et al., 2024). Didden (2001) even found epigeics to be absent from tilled arable soil. However, Van Capelle et al. (2012) and Pelosi et al. (2014) similarly found no relation between epigeic abundance and tillage. Pelosi et al. (2014) stated that tillage may select for functional traits rather than ecological categories. Additionally, some epigeics may have managed to adapt to the conditions under the various treatments through plasticity in their feeding behaviour (Curry & Schmidt, 2007). Treatment may have had a short-term effect, but the epigeic community recovered within the six months of the field experiment.

Endogeic earthworms showed a population decrease in response to soil disturbance and soil bareness. In a global meta-analysis, Briones and Schmidt (2017) report that, although all three ecological groups respond negatively to tillage, epigeic and anecic earthworms are generally affected most. By contrast, several authors describe increased endogeic earthworm abundance after tillage, attributed to incorporation of food (Chan, 2001; Capowiez, Cadoux, Bouchant et al., 2009; Rosas-Medina et al., 2010; Lemtiri et al., 2018) or through alleviation of compaction (Wyss & Glasstetter, 1992), mostly in arable systems. The fact that we removed the grass sod before tillage was applied may explain the discrepancy between these findings and ours. In this set-up, no extra organic matter was incorporated into the soil during tillage. In previous research Chan, 2001; Capowiez, Cadoux, Bouchant et al., 2009; Rosas-Medina et al., 2010; Lemtiri et al., 2018), the negative effects of tillage on endogeic abundance may have been overruled by the benefits of the additional food supply.

We hypothesised a strong effect of the treatments on anecic earthworms, but this was not confirmed (Table 3.4 and 3.5). The difference between NoTillBare (27 ind.

m^{-2}) and TillBare (7.1 ind. m^{-2}) in year 2 was the only significant difference found (Table 3.5). This was surprising, as negative effects of tillage on anecic abundance have been shown in field studies (Pelosi et al., 2014; Torppa & Taylor, 2022), a review (Chan, 2001) and a meta-analysis (Briones & Schmidt, 2017). This may indicate that factors other than tillage and soil bareness played a role. The weak effect of tillage on anecic earthworms in the first year of the experiment may have been due to the dry spring (Table 3.3), causing *L. terrestris* to retreat deeper into its burrow to escape the risk of desiccation. As the soil was tilled only to a depth of 20-25 cm, there may have been little chance of death through mechanical damage. Additionally, six months is a relatively short period compared with most of the field experiments mentioned earlier, which had been established for multiple years (Van Eekeren et al., 2008; Ponge et al., 2013; Pelosi et al., 2014; Torppa & Taylor, 2022). It is possible that the *L. terrestris* population would have declined in the longer term. Overall, the population density of *L. terrestris* was low in this experiment, with 27 ind. m^{-2} being the maximum mean (Table 3.5), whereas the other studies reported 50-88 ind. m^{-2} (Van Eekeren et al., 2008; Ponge et al., 2013; Pelosi et al., 2014; Torppa & Taylor, 2022). This could indicate that the conditions of the grassland were generally unfavourable for *L. terrestris* (Van de Logt, Van der Sluijs et al., 2023a), possibly interfering with the effects of the treatments.

4.4 Not all earthworm species respond similar to tillage and soil bareness

We expected to find a decrease in earthworm species diversity along the intensity gradient of the treatments, in line with existing literature (Wyss & Glasstetter, 1992; Ivask et al., 2007; Capowiez, Cadoux, Bouchant et al., 2009; Sutri et al., 2024), with NoTillGrass having the highest diversity, NoTillBare and TillGrass having intermediate diversity, and TillBare showing the lowest diversity. Instead, Shannon diversity indices did not show significant treatment effects (Supplementary Table S3.1). Most research on this topic was conducted in farming systems that were either arable or ley-arable, both of which are distinct from permanent grasslands in terms of earthworm community (Mazur-Pączka et al., 2019) (Hoeffner, Hotte et al., 2021). In this study, species diversity was resilient to tillage and six months of soil bareness, and this is likely because no species is so vulnerable to tillage that it completely disappears after one application, and generally a period of six months will allow for a recovery of the population.

We observed some species-specific responses to the treatments. Unlike the other species, *L. rubellus*, *A. rosea* and *A. caliginosa* were all more abundant in NoTillGrass than in TillBare. This aligns with the results at the level of the total community and the ecological groups, as these three species represent three of the four generally most abundant species. *A. limicola*, the second most abundant species, showed no response to tillage and soil bareness. We cannot explain this, as species-specific information about its ecology is absent. We only know that this species is mostly associated with agricultural habitats (Ashwood et al., 2024). The low abundance of the remaining species does not provide insight into their responses to the treatments. As for *L. rubellus*, *A. rosea* and *A. caliginosa*, each species seems to show resilience against one negative impact (either tillage or soil bareness), but the combined negative effects decimate their populations, probably due to mechanical damage and decreased food supply.

5. Conclusions

The abundance and biomass of a resident earthworm community was affected by tillage and soil cover loss. It was shown that permanent grassland is the most preferable scenario for sustaining the earthworm community, whereas the combination of tillage and the absence of soil cover most strongly decreased the earthworm population. Tillage had a stronger negative effect on the earthworm community than soil cover loss. This study clearly shows that different grassland renewal strategies have different effects, and that it takes the earthworm community more than six months to recover, even if the practice is applied only once. Particularly endogeic abundance decreased under tillage and soil bareness, showing that this group does not benefit from tillage if no additional organic matter (e.g. the grass sward) is incorporated into the soil. The effect of tillage and soil bareness on epigeics and anecics was less clear, as these groups were less abundant and their treatment response was year-dependent, which is probably due to differences in weather conditions. In conclusion, adjusting grassland management for maintaining earthworm populations requires a comprehensive understanding of the interplay of factors such as land-use, type of tillage, weather conditions and vegetation cover. In the future, more on-farm research, preferably long-term and on different soil types, is needed to improve our understanding of earthworm community dynamics under various grassland renewal strategies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data availability

Data will be made available on request.

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6. Supplementary materials

Supplementary Table S3.1 Overview of the abundance per species per treatment. Significant differences within species between treatments are indicated with letters at the 5% confidence interval. No letters were assigned to *Lumbricus castaneus* and *Lumbricus terrestris* as these species showed an interaction effect (treatment * year). *Octolasyon lacteum* and *Aporrectodea limicola* were only found in year 2 and year 1 respectively. Bottom line shows Shannon Diversity Index per treatment and per year.

		Treatment				Year				
Group	**Unit**	**No till, grass**	**No till, bare**	**Till, grass**	**Till, bare**	**1**	**2**	**Treat-ment (T) p-value**	**Year (Y) p-value**	**T*Y p-value**
L. castaneus	$n*m^{-2}$	11	3.9	7.8	2.1	4.8	7.8	**	0.074	*
L. rubellus	$n*m^{-2}$	29 a	22 ab	14 ab	9.2 b	15 a	22 a	**	0.112	0.381
A. rosea	$n*m^{-2}$	68 a	44 a	23 ab	10 b	19 a	55 b	***	***	0.215
A. caliginosa	$n*m^{-2}$	156 a	89 ab	53 bc	36 c	47 a	120 b	***	***	0.897
O. lacteum	$n*m^{-2}$	16	0	2.8	0	4.6	n.a.	0.103	n.a.	n.a.
A. limicola	$n*m^{-2}$	48	69	40	23	n.a.	45	0.747	n.a.	n.a.
L. terrestris	$n*m^{-2}$	16	16	16	7.1	9.0	18	*	***	*
A. nocturna	$n*m^{-2}$	4.6 a	1.8 ab	0.4 b	1.8 ab	1.9	2.3	*	0.778	0.358
Shannon index		1.37	1.28	1.40	1.14	1.53	1.51	0.148	0.258	



Chapter 4

The anecic earthworm *Lumbricus terrestris* can persist after introduction into permanent grassland on sandy soil

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Abstract

Grasslands are important water-regulating agro-ecosystems. Their ability to store and retain water is of vital importance under the current trend of increasing peak rains and droughts, which are events that can result in economic damage to infrastructure and crops. The presence of the anecic earthworm *Lumbricus terrestris* has a positive effect on the water regulation of the soil. This earthworm creates semi-permanent burrows with a depth of up to 2 m, which increase the water infiltration rate and capacity of the soil. The burrows can also facilitate plant root penetration into deeper soil layers, thus increasing drought resistance. The objective of this research was to explore the ability of *L. terrestris* to survive and reproduce after introduction into grasslands on sandy soils where they had been absent.

We introduced *L. terrestris* into mesocosms on sandy soil under permanent grassland at two dairy farms in the Netherlands. Results showed that while *L. terrestris* was able to survive and produce cocoons, survival rate was low (32% after seven months, 6% after fifteen months) and the number of juveniles was low (2.6 and 2.7 ind. m-2 after seven and fifteen months, respectively). The causes of this low survival rate may be related to the life history of the earthworms introduced, the effects of soil moisture, interspecific competition for food with the native population of epigeic earthworms, and the risk of predation.

We conclude that *L. terrestris* can survive and produce cocoons after introduction into permanent grassland on sandy soil, but only further experimental trials over greater time spans and in non-enclosed plots will determine whether there is realistic potential for *L. terrestris* to develop a stable population.

Keywords: Lumbricidae; earthworm inoculation; mesocosm; water regulation; ecosystem services; interspecific competition

Highlights

- *L. terrestris* can survive 15 months after introduction into grassland on sandy soil
- *L. terrestris* can produce cocoons after introduction into grassland on sandy soil
- *L. terrestris* can survive and produce cocoons after introduction into disturbed sandy soil
- Interspecific competition with *L. rubellus* may hinder *L. terrestris* establishment

1. Introduction

Peak rains and droughts (Intergovernmental Panel On Climate Change, 2014; Lenderink et al., 2011; Lenderink & Van Meijgaard, 2008; Lenderink & Van Meijgaard, 2010) have detrimental effects on both agriculture and society as a whole. If the water infiltration capacity of the soil does not match the amount of precipitation, waterlogging and surface runoff can occur, which result in economic damage to infrastructure and crops. Drought creates problems for drinking water supply, and in recent years it has had negative effects on European crop and grass production (Beillouin et al., 2020; Fu et al., 2020; Van Oort et al., 2023). A trend of decreasing soil moisture in agricultural soils is expected to continue (Grillakis, 2019; Samaniego et al., 2018). The higher sandy soils in the Netherlands are sensitive to drought as they fail to retain water, which subsequently increases problems in lower areas that are sensitive to flooding. The different stakeholders in these areas, such as farmers, drinking water companies and water boards, are looking for measures to increase water infiltration, to enhance water-holding capacity and to decrease drought sensitivity.

Earthworms are known to provide water-regulating ecosystem services. They can increase the hydraulic conductivity, water infiltration capacity and water-holding capacity of the soil (Bouché & Al-Addan, 1997; Capowiez, Cadoux, Bouchant et al., 2009; Clements et al., 1991; Deru et al., 2018; Edwards et al., 1990; Hallam & Hodson, 2020; Hoogerkamp et al., 1983; Ligthart & Peek, 1997; Spurgeon et al., 2013; Stockdill, 1966). Three major ecological groups of earthworms can be distinguished: epigeic, endogeic and anecic (Bouché, 1977). The anecic species *Lumbricus terrestris* is particularly suited for the objective of water regulation because of its ability to penetrate compacted soil layers (Capowiez, Cadoux, Bouchant et al., 2009) and to create semi-permanent (Rushton, 1986) vertical burrows that can reach a depth of > 2 m (C. A. Edwards & Bohlen, 1996), facilitating both water flow and root growth into deeper soil layers (C. A. Edwards & Lofty, 1978).

L. terrestris has been found in abundance in grasslands in several parts of the world (Boag et al., 1997; Daniel, 1992; Kruuk et al., 1979; Steckley, 2020). A review by Eijsackers (2011) showed that *L. terrestris* dispersal was highest in grassland on fertile mineral soil. However, a national inventory among Dutch dairy farms revealed the presence of anecic earthworms in the grasslands of only 21% of the Dutch dairy farms studied. These earthworms belonged to two species, namely *Aporrectodea longa* and *L. terrestris*, the latter being more common (Van Eekeren et al., 2014).

The low prevalence of anecic earthworms was somewhat surprising and may result from the frequent grassland renewal of the last 40 years, combined with arable crop rotation and the associated intensive soil cultivation, such as ploughing. Anecic earthworms are known to be sensitive to soil cultivation and rotation of grassland with arable crops (Chan, 2001; Chan, 2004; Fox et al., 2017; Van Eekeren et al., 2008).

Since the natural dispersal of *L. terrestris* is slow (Ligthart & Peek, 1997; Nuutinen et al., 2006; Nuutinen et al., 2011), a number of research projects investigated whether it is possible to inoculate soils with this species. Matters like survival rate (Andriuzzi et al., 2015; Butt, 1999; Forey et al., 2018), dispersal (Grigoropoulou & Butt, 2010; Hoogerkamp et al., 1983; Ligthart & Peek, 1997), the effect of management (Frazão et al., 2019; Grigoropoulou & Butt, 2010; Kautz et al., 2014; Nuutinen et al., 2011) and experimental set-up (Butt, 2008) were addressed. Various lessons can be learned from these studies. Butt (2011a) compared different methods for introduction and introduced the Earthworms Inoculation Unit (EIU). Grigoropoulou and Butt (2010) found that individuals that were collected on-site had a higher chance of survival than commercially-bought *L. terrestris*. Hoogerkamp et al. (1983) studied introduction and dispersal in Dutch polders with clayey soil, and they found that *L. terrestris* prefers a low water table, as flooding can damage its burrow and leave it without oxygen. Both interspecific and intraspecific competition have been reported to negatively affect the survival and growth of *L. terrestris*, underlining the importance of interaction with the native earthworm population (Keith et al., 2018; Lowe & Butt, 2002; Lowe & Butt, 2005; Shuster et al., 2003). Nuutinen et al. (2011) emphasised the importance of field margins and long-term monitoring. The species was found to be able to burrow through compact soil layers (Joschko et al., 1989), although it displayed a preference for less compacted soil (Ducasse et al., 2021; Jégou et al., 1999).

However, some questions remain unresolved. For example, the requirements for *L. terrestris* to survive introduction and to start reproducing are quite poorly understood, high earthworm mortality in field experiments is regularly reported (Andriuzzi et al., 2015; Butt, 1999; Forey et al., 2018), and establishment and dispersal rates can be exceedingly low (Ligthart & Peek, 1997; Nuutinen et al., 2006; Nuutinen et al., 2011). Furthermore, research on *L. terrestris* introduction has mainly focused on clay and loamy soils. We argue that *L. terrestris* introduction in grasslands on sandy soils needs attention, as it is a common soil type, which is sensitive to both peak rains and drought. *L. terrestris* is known to already occur in some sandy soils (Chamberlain &

Butt, 2008; Van Eekeren et al., 2014), and this work aimed to explore the possibility of introducing this earthworm into grasslands where they are currently absent.

To this end, we introduced *L. terrestris* into mesocosms in permanent grasslands on sandy soils on two dairy farms: a conventional farm with a history of artificial fertiliser and slurry application, and an organic farm with a history of farmyard manure use. We harvested half of the mesocosms after seven months and re-inoculated them, and after fifteen months all the mesocosms were harvested. The re-inoculated mesocosms served as an *explorative experiment* to see how *L. terrestris* would react to less compact disturbed soil without a resident earthworm population. Our aim was to determine whether introduced *L. terrestris* would be able to survive and reproduce in the protective and semi-controlled environment of this mesocosm set-up. We intended to provide a proof of principle, not to mimic actual field conditions. We hypothesised (i) that *L. terrestris* is able to survive introduction into permanent grassland on sandy soil, and (ii) that *L. terrestris* is able to reproduce after introduction into permanent grassland on sandy soil. In our explorative experiment we expected to find (i) that *L. terrestris* would be able to survive and reproduce on disturbed soil without a resident population, (ii) that *L. terrestris* would dig deeper burrows in disturbed (less compact) soil, and (iii) that *L. terrestris* would settle more successfully in the absence of a competing resident earthworm population.

2. Material and methods

2.1 Study area

On the 5th of April 2019, a field experiment was installed in two permanent grasslands on sandy soils (Haploquod [USDA]) in the Netherlands. One grassland was located on a conventional farm (location A, 52°32’05.0”N 6°30’19.7”E) while the second was located on an organic farm (location B, 52°05’25.6”N 5°35’33.3”E). Both grasslands were grazed by dairy cows and were dominated by *Lolium perenne*. A baseline assessment of the selected grasslands had previously demonstrated absence of *L. terrestris*. On the 3rd of May 2018 (Location B; this location was sampled by Salánki and De Goede (2020) and the 11th of September 2018 (location A), four soil blocks of 20x20x20 cm were dug out and carefully hand-sorted. Samples were taken at representative and evenly distributed locations within these grasslands. After each soil block was dug out, 1-2 l of AITC (allyl isothiocyanate) solution was applied to the pit and for 30 minutes it was checked whether earthworms surfaced. On no occasion

L. terrestris was detected. Soil organic matter was higher at location B, whereas other soil chemical properties and texture were comparable (Table 4.1). In the 10-40 cm soil layer, soil penetration resistance was higher at location B, whereas in the 40-50 cm soil layer, penetration resistance was higher at location A (Suppl. Figure S4.1).

Table 4.1 Soil properties in the 0-10 cm soil layer and management for location A and location B

	Location A	**Location B**
Soil type (USDA)	Haploquod	Haploquod
Chemical properties		
pH	5.9	5.5
Organic matter (%)	4.3	7.4
N-total (g N/kg dry soil))	2.3	4.4
P-Al (mg P2O5/100 g)	55	55
Soil texture		
% sand	88	84
% clay	< 1	1
% silt	8	8
Management		
Cutting	Yes	No
Grazing	Yes	Yes
Fertilisation	Yes	Yes
	Slurry manure + artificial fertiliser	Farmyard manure

2.2 *Lumbricus terrestris* introduction into permanent grassland (Experiment 1)

Twenty mesocosms were installed per location. Before installation, grass was mowed to a height of 4 cm. With a crane, 20 steel pipes (ø 61 cm, height 50 cm) were driven 40 cm into the soil, leaving a 10 cm ring above the soil surface. Pipes were spaced 90 cm apart (Suppl. Figure S4.2). Ten mesocosms were assigned to be harvested after seven months and ten mesocosms were assigned to be harvested after fifteen months. In both groups, five mesocosms received 15 adult *L. terrestris* individuals each, 51 ind. m^{-2}, which is the same inoculation density as used by Forey et al. (2018). We chose this rather high density as we expected that not all earthworms would survive. The other five mesocosms served as controls, and no earthworms were added to these. Earthworms were purchased from a commercial supplier (https://www.thedutchnightcrawlers.nl/) that had imported them from Canada, and individuals with a clitellum were considered adult. The average weight per earthworm at the moment of introduction was 4.03 g.

To increase the chances of successful introduction, each mesocosm received 3 mm of water. An auger was used to loosen the top 2 cm of the soil in three places, so as to facilitate earthworm burrowing without damaging the whole grass sod. Each mesocosm received 50 g of freshly cut grass from the site and was then covered with a moist burlap cloth which was removed after five days. All mesocosms were covered with 1.5 mm mesh gauze to prevent the earthworms from escaping aboveground and to protect them against predators (Suppl. Figure S4.2). Escape belowground was considered highly unlikely, as *L. terrestris* burrows vertically and disperses over the soil surface (Mather & Christensen, 1988).

On the 23rd of April 2019, the grass in and around the pipes was mowed to a height of 4 cm, and 10 mm of water and 300 g of farmyard manure (collected at location B) was applied to the mesocosms (316 g dry matter kg^{-1}; 6.6 g total N kg^{-1}; 0.9 g NH3-N kg^{-1}; 5.8 g N-org kg^{-1}). Subsequently, the sites were mown in and around the pipes every four weeks, to prevent the grass from growing into the mesh gauze covers. After mowing, the grass cuttings were removed from the mesocosms. In July 2019, all mesocosms received 20 mm of water because of the exceptionally dry summer. In addition to the water we applied, the farmer at location A irrigated the grassland three times with 22.5 mm of water during the growing season of 2019.

In November 2019, seven months after inoculation, five inoculated mesocosms and five controls were harvested at each location, so as to determine whether the *L. terrestris* introduced had been able to survive and produce cocoons in the short term. The harvesting procedure was as follows: in situ, the soil was removed from the mesocosms with a spade. This was done in three layers (0-20 cm, 20-40 cm and 40-60 cm depth), and each soil layer was collected in a separate container. It is important to note that *L. terrestris* can move rapidly through its burrows, especially when disturbed, like during the harvesting process. The depth at which we found the earthworms was therefore considered the *minimum* depth that they had reached since inoculation. The soil layers were hand-sorted on-site, to collect all earthworms. The hand-sorted soil layers were returned to the mesocosms in their original order. The collected earthworms were rinsed, weighed and preserved in alcohol. The earthworms were classified as adults or juveniles and identified to species level if possible (Sims & Gerard, 1985; Stöp-Bowitz, 1969). The other mesocosms were left untouched. In February 2020, 600 g of farmyard manure was applied to each of the unharvested mesocosms, at both locations. In July 2020, 15 months after inoculation, all mesocosms were harvested following the same procedure as in November 2019.

2.3 *Lumbricus terrestris* introduction into disturbed soil without resident earthworms population (Experiment 2)

After sorting in November 2019 (Experiment 1), the soil was restored in the mesocosms. All three layers (0-20, 20-40 and 40-60 cm) were put back in their original order. Subsequently, the five mesocosms previously allotted to inoculation during Experiment 1 once again received 15 *L. terrestris* (51 ind. m^{-2}) each, following the same procedure as in April 2019. The five control mesocosms again served as controls in Experiment 2, to ensure that no *L. terrestris* cocoons were present. Perennial ryegrass (*Lolium perenne*) was seeded and a layer of leaf litter (mainly *Aesculus hippocastanum*) was added to the mesocosms to provide a food source and a protective layer for the earthworms. In February 2020, the remaining leaf litter was removed and 900 g of compost and 30 g of concentrates (composed of maize, barley, wheat, nutricell, pulp and minerals [900 g dry matter kg^{-1}; 12.8 g total N kg^{-1}]) was applied to the surface. These concentrates were provided as an additional protein source to support the earthworms (personal communication with the Dutch Nightcrawlers, earthworm supplier). Harvesting took place after eight months in July 2020 (Figure 4.1), using the same procedure as in November 2019 and July 2020 for Experiment 1.

2.4 Weather data

Soil temperature data was obtained from the nearest weather stations and can be found in Suppl. Table S4.1 and S4.2, and monthly rainfall and air temperature per location can be found in Suppl. Figure S4.3 and S4.4. Generally, 2019 and 2020 were relatively dry, especially July (2019 and 2020) and August (2019). April and May of 2020 were exceptionally dry, with little precipitation for two months. Temperatures were close to the long-term average, with January and February 2020 being slightly warmer than average.

2.5 Statistical analysis

GenStat software was used to perform a three-way ANOVA on the data collected. Factors were location (A and B), treatment (control and *L. terrestris*) and harvest (November 2019 and July 2020). Experiment 1 and Experiment 2 were treated as two separate experiments. LSDs were determined for the interpretation of two- and three-way interactions.

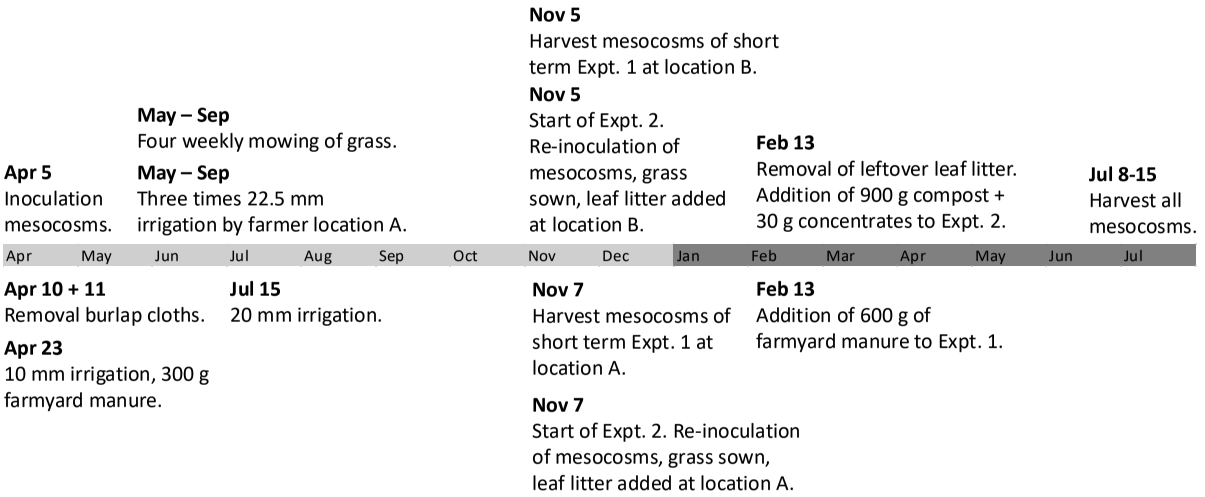


Figure 4.1 Timeline of the field experiment. Light grey: 2019, dark grey: 2020. When location is not mentioned explicitly, the intervention was executed at both locations.

3. Results

3.1 Introduction into undisturbed soil (Experiment 1)

3.1.1 Anecic earthworms (Experiment 1)

Anecic species were represented by *L. terrestris* (22%) and *Aporrectodea nocturna* (78%) at location A and by *L. terrestris* (100%) at location B. The number of adult *L. terrestris* (m^{-2}) was significantly affected by treatment and harvesting date, but not by location (Table 4.2). Seven months after introduction (November 2019), *L. terrestris* abundance was significantly higher than it was after fifteen months (July 2020) (Table 4.2; Figure 4.2). After seven months, 32% of the density initially introduced was recovered, and after fifteen months 6% was recovered.

The total *L. terrestris* biomass (g m^{-2}) was significantly affected by both treatment and harvesting date, but not by location. The interaction effect of harvesting date and treatment was the only one yielding significance (Table 4.2). We measured 48 g m^{-2} of *L. terrestris* biomass in inoculated mesocosms in November 2019 (after seven months), and 12 g m^{-2} of *L. terrestris* biomass in July 2020 (after fifteen months). The average weight per *L. terrestris* that was harvested intact was 2.8 g in November 2019 and 4.2 g in July 2020.

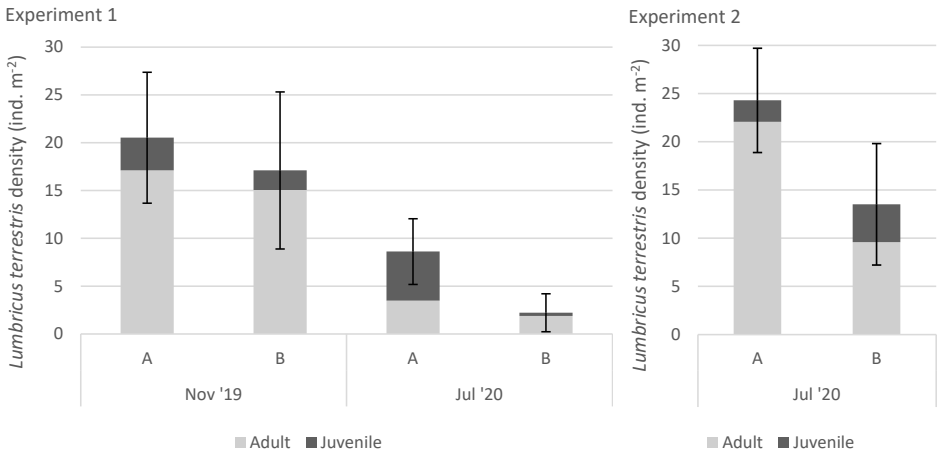


Figure 4.2 Experiment 1, undisturbed soil; Experiment 2, disturbed soil. Density of *Lumbricus terrestris* at locations A and B in inoculated mesocosms. Harvested in November 2019 and July 2020. Bars indicate average deviation of total number *L. terrestris* (adult and juvenile combined).

Table 4.2 Earthworm abundance (ind. m^{-2}) and biomass (g m^{-2}) for *Lumbricus terrestris*, epigeics and endogeics in undisturbed soil (Experiment 1). Location A = conventional dairy farm; location B = organic dairy farm; Harvest 1 = November 2019; Harvest 2 = July 2020; Treatment C = control; Treatment Lt = inoculation with 15 *L. terrestris* (51 ind. m^{-2}). HxT is the interaction effect between harvesting date and treatment. Other interactions (H x L, H x L and H x L x T) were not significant. Numbers in bold indicate significance. P-values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant.

	Means						p-value			
	Treatment (T)		Location (L)		Harvest (H)		T	L	H	H x T
	C	Lt	A	B	2019	2020				
Abundance (ind. m^{-2}) in 0-60 cm soil layer										
Total	619	588	587	620	**465**	**742**	ns	ns	***	ns
Epigeic	177	153	**121**	**208**	**136**	**194**	ns	**	*	ns
Endogeic	416	395	422	388	**295**	**515**	ns	ns	***	ns
Anecic	**30**	**14**	**30**	**14**	22	22	**	**	ns	ns
L. terrestris	**1.8**	**12**	8.5	5.7	**11**	**3.4**	***	ns	***	**
Adults	**0.8**	**9.5**	5.9	4.5	**8.5**	**1.8**	***	ns	**	**
Juveniles	1.0	2.7	2.5	1.2	2.2	1.5	ns	ns	ns	ns
Biomass (g m^{-2}) in 0-60 cm soil layer										
Total	**134**	**162**	151	145	147	149	*	ns	ns	ns
L. terrestris	**2.5**	**30**	19	13	**26**	**6.6**	***	ns	**	**
Adults	**1.9**	**27**	17	12	24	4.2	***	ns	**	**
Juveniles	**0.6**	**3.3**	2.1	1.7	1.5	2.4	**	ns	ns	ns
Non-*L. terrestris*	132	133	132	133	122	143	ns	ns	ns	ns

The vertical distribution of *L. terrestris* over the three different soil layers at the first harvest indicated a discrepancy between locations A and B. At location A, 77% of the *L. terrestris* were found in the upper 20 cm of the soil, whereas this was only 44% at location B. After fifteen months, the proportion of *L. terrestris* found in the 20-40 and 40-60 layers had increased at both locations (Figure 4.3).

3.1.2 Non-anecic earthworms (Experiment 1)

Total earthworm abundance was significantly higher in July 2020 than it was in November 2019 (means of 742 and 465 m^{-2}, respectively) (Table 4.2). The total earthworm abundance was affected neither by location nor by treatment.

The inoculated mesocosms yielded a significantly higher total earthworm biomass (g m^{-2}) than the controls (162 g versus 134 g m^{-2}) (Table 4.2) as a consequence of the added *L. terrestris*. The non-*L. terrestris* biomass (g m^{-2}) was not significantly affected by treatment, location or harvest (Table 4.2).

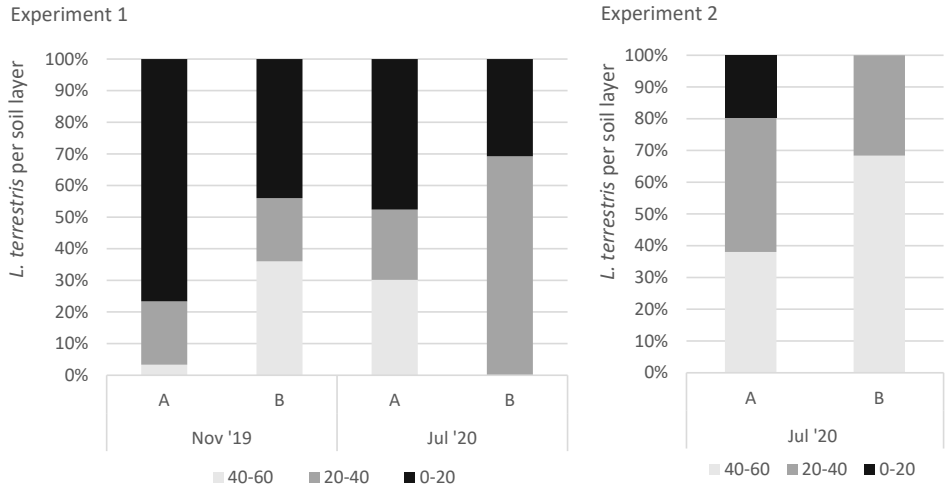


Figure 4.3 Experiment 1, undisturbed soil; Experiment 2, disturbed soil. Distribution of *Lumbricus terrestris* in inoculated mesocosms over three soil layers (0-20; 20-40 and 40-60 cm) at locations A and B, harvested in November 2019 and July 2020.

Across all treatments, epigeic species were represented by *Lumbricus rubellus* (98.9%), *Lumbricus castaneus* (0.8%) and *Dendrobaena octaedra* (0.3%) at location A, and by *L. rubellus* (83.6%), *L. castaneus* (13.5%), *Dendrobaena rubida* (2.6%) and *D. octaedra* (0.3%) at location B. Epigeic earthworm abundance was significantly affected by location, but not by treatment or harvesting date. The difference was non-significant, but on average, the inoculated mesocosms contained fewer epigeic worms than the control mesocosms at both locations and both harvesting dates (Figure 4.4). The largest numerical difference between the number of epigeic earthworms in inoculated mesocosms and control mesocosms was observed in November at location B, with means of 136 and 194 ind. m^{-2}, respectively.

Endogeic species were represented by *Allolobophora chlorotica* (97.3%) and *Aporrectodea caliginosa* (2.7%) at location A and *A. caliginosa* (100%) at location B. Endogeic earthworm abundance was affected by harvesting date, but not by treatment or location (Table 4.2).

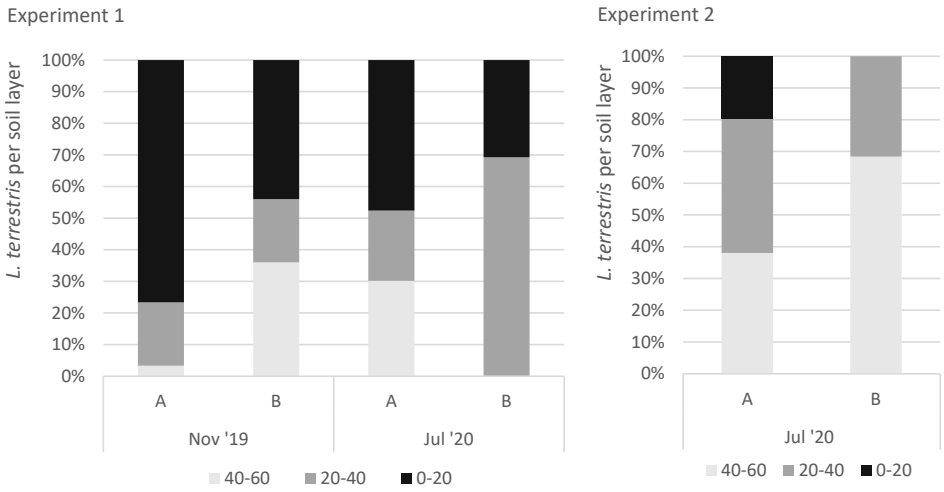


Figure 4.4 Experiment 1, undisturbed soil; experiment 2, disturbed soil. Mean epigeic earthworms density at locations A and B. C = control; Lt = inoculated with 15 *Lumbricus terrestris* (51 ind. m^{-2}). Bars indicate average deviation of total number epigeic earthworms (adult and juvenile combined). The star indicates a significant difference between the number of epigeic worms in C and Lt mesocosms at location B.

3.2 Introduction into disturbed soil without resident earthworm population (Experiment 2)

3.2.1 Anecic earthworms (Experiment 2)

Anecic species were represented by *L. terrestris* (47%) and *A. nocturna* (53%) at location A and *L. terrestris* (100%) at location B. We recovered 33% of the *L. terrestris* introduced (Table 4.3). Both treatment and location had a significant effect on *L. terrestris* abundance. Inoculation resulted in an average of 19.4 *L. terrestris* m^{-2} after eight months, mostly adults (84%). The interaction effect of location and treatment yielded significant differences. More *L. terrestris* were found in inoculated mesocosms at location A than at location B, namely 26 m^{-2} versus 12.8 m^{-2} (Figure 4.2). The two locations differed significantly in abundance for adult *L. terrestris*, but not for juveniles. In inoculated mesocosms at location A, 23.4 m^{-2} adults and 2.6 m^{-2} juveniles were found, meaning that 11% of the population was juvenile. At location B, 10.4 m^{-2} adults and 2.4 m^{-2} juveniles were found, which means that 23% of the population was juvenile.

Table 4.3 Earthworm abundance (ind. m-2) and biomass (g m^{-2}) *Lumbricus terrestris*, epigeics and endogeics in Experiment 2 (disturbed soil); Location A = conventional dairy farm; location B = organic dairy farm; Treatment C = control (no added *L. terrestris*); Treatment LT = 15 added *L. terrestris* (51 ind. m^{-2}). Significant values at the confidence interval of $p < 0.05$ in bold. P-values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant.

	Means				**p-value**		
	Treatment		**Location**				
	C	**LT**	**A**	**B**	**T**	**L**	**T x L**
Abundance (ind. m^{-2}) in 0-60 cm soil layer							
Total	**525**	**390**	**541**	**374**	*	**	*
Epigeic	**178**	**106**	**92**	**191**	*	**	ns
Endogeic	332	264	**432**	**165**	ns	***	ns
Anecic	14	21	21	14	ns	ns	*
L. terrestris	**0.3**	**19**	**13**	**6.7**	***	*	*
Adults	**0**	**17**	**12**	**5.2**	***	*	*
Juveniles	**0.3**	**2.5**	1.3	1.5	*	ns	ns
Biomass (g m^{-2}) in 0-60 cm soil layer							
Total	112	133	129	117	ns	ns	ns
L. terrestris	**0.5**	**73**	46	27	***	ns	ns
Adults	**0**	**70**	45	25	***	ns	ns
Juveniles	0.5	2.8	1.3	2	ns	ns	ns
Non-*L. terrestris*	**112**	**60**	83	90	**	ns	ns

The total *L. terrestris* biomass (g m^{-2}) was significantly affected by treatment, but not location (Table 4.3). The average weight of the recovered *L. terrestris* that were harvested intact was 3.9 g per individual.

The distribution of *L. terrestris* over the three different soil layers appeared to differ between the locations. At location A, 20% of the *L. terrestris* was found in the 0-20 cm soil layer, whereas at location B, 100% of the *L. terrestris* was found in the lower 20-60 cm of the soil (Figure 4.3).

3.2.2 Non-anecic earthworms (Experiment 2)

Treatment, in addition to location, had a significant effect on the total earthworm abundance (Table 4.3). Control mesocosms yielded significantly more earthworms than inoculated mesocosms (means of 525 and 390 m^{-2}, respectively) (Table 4.3). The total earthworm abundance was significantly higher at location A than at location B (means of 541 and 374 ind. m^{-2}, respectively) (Table 4.3).

The presence of *L. terrestris* had a significant impact on the biomass dynamics of the other earthworms. The non-*L. terrestris* part of the earthworm biomass was almost halved under the inoculation treatment (means of 112 g m^{-2} for controls and 60 g m^{-2} under inoculation treatment) (Table 4.3). The contribution of *L. terrestris* to the total earthworm biomass in the inoculated mesocosms masked the distinction in total biomass between inoculated and control treatments.

Epigeic species were represented by *L. rubellus* (99.5%) and *L. castaneus* (0.5%) at location A and *L. rubellus* (95.6%) and *D. rubida* (4.4%) at location B. Treatment as well as location had a significant effect on epigeic earthworm abundance. Inoculated mesocosms contained significantly fewer epigeics than the controls (means of 106 and 178 m^{-2}, respectively) (Table 4.3; Figure 4.4). Location A harboured significantly fewer epigeics than location B (means of 92 and 191 m^{-2}, respectively) (Table 4.3; Figure 4.4).

Endogeic species were represented by *A. chlorotica* (97.3%) and *A. caliginosa* (2.7%) at location A, and by *A. caliginosa* (100%) at location B. The number of endogeic earthworms was significantly affected by location, but unlike the epigeic earthworms it was not significantly affected by treatment. More endogeics were found at location A than at location B (means of 432 m^{-2} and 165 m^{-2}, respectively) (Table 4.3).

4. Discussion

4.1 *Lumbricus terrestris* survival

We hypothesised that *L. terrestris* is able to survive introduction into a permanent grassland on sandy soil where it is currently absent. Indeed, we retrieved adult *L. terrestris* individuals from the mesocosms at both locations, which shows that they can survive after introduction. As it can take *L. terrestris* over one year to mature under field conditions (Daniel, 1992), we assume that the adult *L. terrestris* earthworms that we retrieved were the earthworms that we had initially introduced and not a second generation.

Unexpectedly, we also encountered some *L. terrestris* individuals in control mesocosms. It might be that somehow earthworms managed to escape and colonise a neighbouring mesocosm. Another explanation could be that the morphological species determination was inaccurate for these individuals. Although the determination was carried out by an expert, earthworms can be affected from the harvesting

process or display some degree of phenotypic plasticity (Domínguez & Velando, 2013) which complicates morphological species determination.

Overall, the survival rate was lower than we had expected. From the undisturbed mesocosms of Experiment 1, we retrieved 32% of the *L. terrestris* earthworms after seven months and only 6% after fifteen months. From the disturbed soil mesocosms of Experiment 2, we retrieved 33% after eight months. This is in contrast to our prediction that disturbed soil – which is less compact and easier to burrow through – and the absence of a resident earthworm population would promote *L. terrestris* survival rate.

The survival rate we observed is in line with the findings of Andriuzzi et al. (2015). They retrieved 40%, eight months after *L. terrestris* introduction into mesocosms on a conventionally managed arable field on sandy-loamy soil. Possible explanations for the low survival rate could be the life history of the earthworms introduced, soil moisture, interspecific competition, and predation; these are discussed in the following sections.

4.1.1 Life history of the Lumbricus terrestris introduced

L. terrestris mortality may have been related to their origin and life history. As mentioned before, the earthworms were harvested and collected in Ontario, Canada. Soils in this area are classified as grey-brown luvisols or orthic and albic luvisols. Alfalfa is the most prevalent crop, and fields are generally fertilised with liquid cow manure (Steckley, 2020). After harvesting, earthworms were stored and subsequently sent to the Netherlands. They were kept for 1-2 weeks in a mixture of compost and were fed a specially composed concentrate (personal communication with 'The Dutch Nightcrawlers') until we received them. We selected mature individuals, i.e. having secondary sexual characteristics (Butt & Nuutinen, 2005). The exact age of the earthworms was unknown.

We cannot know the precise effects of the harvest, travel, storage and introduction procedure on the earthworms. Grigoropoulou and Butt (2010) reported that *L. terrestris* earthworms that were collected on-site performed better than commercially purchased individuals, but they give no explanation for this observation. In accordance with this, Vos et al. (2022) reported that in their experiment on sandy soil, *L. terrestris* from Canada showed limited survival, whereas locally collected A. longa performed better. However, we cannot exclude that this difference relates more to

differences between the suitability of the species than to differences between the life history of the earthworms.

The sudden and repeated change of environment may have weakened the Canadian earthworms. The presence of pathogens or parasites (new to these earthworms) may have been lethal.

4.1.2 Soil moisture

The spring of 2020, one year into the experiment, was particularly dry and warm, causing a risk of death through desiccation. Aestivation is very rarely observed for *L. terrestris* (Gerard, 1967; Potvin & Lilleskov, 2017). In general, the species remains active during periods of drought (Eisenhauer et al., 2008) and applies different strategies to avoid desiccation, such as habitat choice, deep burrowing and possibly cocoon incubation time. Deep burrowing allows the earthworms to retreat to the more stable moisture and temperature regimes of deeper soil layers in times of adverse conditions in the top soil. We think some of the earthworms introduced used this strategy, which may explain why we found a larger proportion of *L. terrestris* in deeper soil layers at location B, where the farmer did not apply irrigation.

4.1.3 Interspecific competition

We expected that competition with resident earthworms would negatively affect *L. terrestris* establishment. Interspecific competition between earthworms is not uncommon (Abbott, 1980; Baker et al., 2002; Winsome et al., 2006). *L. rubellus* (which represented close to 99% of the resident epigeic population in our research) and *L. terrestris* share a similar dietary niche (Shipitalo et al., 1988). Shuster et al. (2002) introduced *L. terrestris* into an arable field. They noticed that under one treatment, *L. terrestris* establishment was very modest (7 ind. m^{-2}) and apparently happened at the expense of *L. rubellus*, with numbers of the latter declining to one-fourth of the original. Under the treatment of higher resource availability and variety, they did not observe this effect, which led them to conclude that scarcity in resources can cause interspecific competition between *L. terrestris* and *L. rubellus*. In our experiment, food may have been a limiting factor, leading to competition between species, which may have limited population growth and maturation of the earthworms. Preferably, weight change of *L. terrestris* inoculated in relation to *L. rubellus* abundance would have been calculated. But unfortunately, only very few *L. terrestris* were harvested intact. The average biomass per individual is

thus based on too few animals to draw conclusions about weight gain or loss after inoculation.

Interspecific competition may also explain the difference in *L. terrestris* survival rate between the two locations. At location B, *L. rubellus* was more abundant than at location A (means of 159 and 110 *L. rubellus* m^{-2}, respectively, possibly because farm B had a history of using farmyard manure, whereas farm A had a history of slurry application. Farmyard manure is known to stimulate epigeic earthworms (Van Eekeren et al., 2009). The higher abundancy of epigeic earthworms may have led to more intense competition for resources and thus to lower *L. terrestris* survival. In the inoculated mesocosms of Experiment 1, we found on average 146 *L. rubellus* m^{-2} at location B and 101 m^{-2} at location A (non-significant difference).

During the first harvest, all earthworms were removed from the mesocosm but the cocoons remained. As location B harboured more *L. rubellus* than location A, it is most likely that more *L. rubellus* cocoons also remained in the soil. In this case, *L. terrestris* possibly suffered from more intense interspecific competition for food, which could explain why we found significantly fewer *L. terrestris* at location B in Experiment 2.

4.1.4 Predation

We suspect that predation by rooks (*Corvus frugilegus*) may have influenced the survival rate of the *L. terrestris* in the mesocosms at location B. Although the mesocosms were covered completely with nets, shortly after the establishment of the experiment, rooks made small holes in the nets. Rooks are known to include *L. terrestris* in their diet (Coleman, 1971). Afterwards, we replaced the damaged nets and set up a scarecrow. The bird damage reduced, but we cannot completely exclude predation. In contrast, at location A we found hardly any traces left by birds trying to reach into the mesocosms. Predation by rooks at location B could also explain why *L. terrestris* was generally found deeper in the soil than at location A; the *L. terrestris* in the 0-20 cm layer may have been predated on or the earthworms may have burrowed deeper to escape from the rooks.

4.2 *Lumbricus terrestris* reproduction

Our second hypothesis was that *L. terrestris* is able to reproduce after introduction into a grassland on sandy soil. The discovery of juveniles confirmed that *L. terrestris* produced cocoons after introduction (Figure 4.2). Unfortunately, due to the relatively short time span of the field experiment, we cannot be sure that the earthworms

mated after inoculation. *L. terrestris* can store sperm and has been observed to produce viable cocoons six months (Butt & Nuutinen, 1998) or even 17 months (Butt, 2011b) after mating had taken place.

We expected that the benefits of a less compacted soil which is devoid of earthworms would lead to increased reproductive output in the mesocosms with disturbed soil without a resident population (Experiment 2). Earthworms that need less time and energy for burrowing invest more in foraging and reproduction (Binet et al., 1997). However, this was not apparent from the results of the explorative study. Experiment 2 did not run simultaneously with Experiment 1, hence we only consider these comparisons as an indication. The density of *L. terrestris* juveniles in inoculated mesocosms was very similar across both experiments and harvesting dates. However, when we compare our findings to previous observations in the laboratory by Butt et al. (1992) 25 ind. year^{-1}; Butt (1993) 40 ind. year^{-1}; Lakhani and Satchell (1970) 3-13 ind. year^{-1}, the number of juveniles that we found was extremely modest (0.2 juvenile per adult in the first harvest of Experiment 1; 1 juvenile per adult in the second harvest of Experiment 1; 0.2 juvenile per adult in Experiment 2).

Several factors may have affected the *L. terrestris* reproductive output. First, drought may have played a role, especially at location B since no irrigation took place there. Previous research has confirmed that soil moisture content influences *L. terrestris* reproduction output, with too little moisture having adverse effects on reproduction (Berry & Jordan, 2001; Butt, 1991; Butt et al., 1992; Daniel et al., 1996). In addition, *L. terrestris* mating takes place on the soil surface. Edwards and Bohlen (1996) describe that *L. terrestris* surfaces at night during periods of considerable rain, although not exclusively under these circumstances (Butt et al., 2003). The dry summer of 2019 and dry spring of 2020 may have prevented *L. terrestris* from exploring the soil surface and encountering a partner. Adverse conditions may have caused a preference for cocoon production through the use of previously (pre-experiment) stored sperm over mating in situ.

Second, the age of the earthworms introduced may have played a role. Unfortunately, we could not know their age, as they were 'wild earthworms'. Previous research indicates that reproduction output decreases as *L. terrestris* ages (Butt, 1991; Daniel, 1991). It is also possible that senescence of the worms limited cocoon output.

Third, considerable variety in the incubation time of *L. terrestris* cocoons has been observed under artificial conditions. Several months is no exception (Butt et al., 1992),

and in extreme cases, incubation can take almost two years, as was demonstrated in a lab experiment (Butt, 2011b; Ducasse et al., 2021). Prolonged incubation time can be used as a strategy to avoid drought for the offspring. This makes it even more complex to quantify reproductive output in the field. We only collected juveniles and not cocoons, as it was practically impossible to collect and identify *L. terrestris* cocoons by hand-sorting the soil from the mesocosms. The earthworms had either produced few cocoons, or the dry conditions during the experiment had extended cocoon incubation time and influenced the detectible reproductive output of the individuals that we had introduced.

Overall, the low number of juveniles could result from low cocoon production, low hatching success, low juvenile survival, extended incubation time, or a combination of these.

4.3 Distribution over soil layers

In agreement with our expectations, a larger proportion of *L. terrestris* was found in deeper soil layers in disturbed soil compared to undisturbed soil (Figure 4.3). However, as the experiments did not run simultaneously, we consider these results as merely an indication. Possibly, less compact soil facilitates deeper burrowing. *L. terrestris* able to burrow through compacted soil (Capowiez, Cadoux, Bouchand et al., 2009), but burrowing activity is known to be negatively correlated to soil bulk density (Rushton, 1986), and deeper burrows were found in less compacted soil (Ducasse et al., 2021; Joschko et al., 1989).

More *L. terrestris* were found deeper than 20 cm at location B than at location A (Figure 4.3). We think this may be due to soil moisture, as location A was irrigated and location B was not. The upper part of the soil is more susceptible to drought, which may have killed earthworms in the top 20 cm layer or forced them to move to deeper soil layers. Moreover, the predation pressure of rooks at location B may have played a role.

5. Conclusion

This mesocosm field study demonstrated that it is possible for *L. terrestris* to survive and produce cocoons after introduction into permanent grassland on sandy soil. It showed that *L. terrestris* can survive and produce cocoons after introduction into disturbed sandy soil from which the resident earthworm population has been

removed. However, these results should be interpreted with care, as the field experiment lasted only 15 months and both survival rate and number of juveniles were low. Our observations indicate that life history, soil moisture, predation, as well as food availability and interspecific competition with epigeics could have led to limited survival and reproduction. Based on our results, we think there is still some potential for *L. terrestris* inoculation into grasslands on sandy soil. However, only further experimental trials over greater timespans and in non-enclosed plots will determine whether *L. terrestris* inoculation can lead to the development of a stable population in permanent grassland on sandy soil. Evidently, reproduction has to be greater than loss if the population is to persist and disperse. We make the following recommendations for future research: collect *L. terrestris* from nearby grasslands that resemble field conditions at the experimental site; monitor soil moisture and soil temperature at the experimental site; keep the individuals from the Canadian inoculum under perfect lab conditions – parallel to the field experiment to monitor survival; provide sufficient suitable food to prevent competition; and run a disturbed and undisturbed soil experiment simultaneously, for better comparison between these treatments.

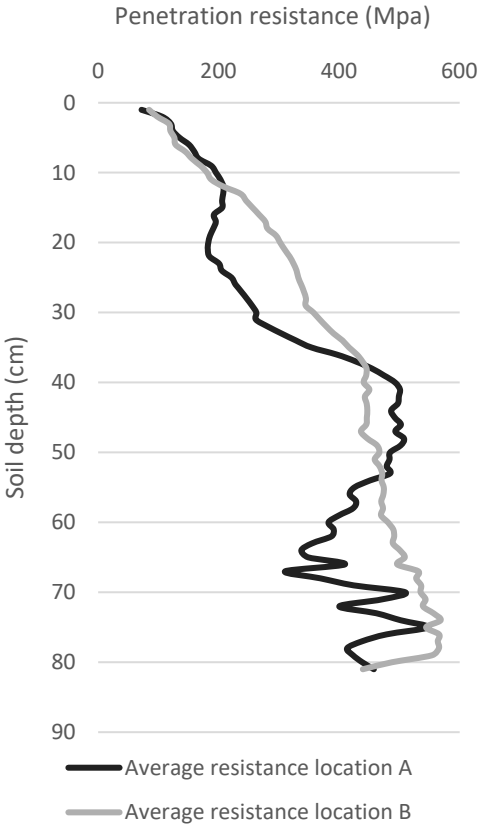
6. Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

7. Acknowledgements

We want to thank the dairy farmers Evert Kremer and Jan Dirk van der Voort for letting us use their grasslands as experimental sites. This research would not have been possible without the very much appreciated help of all the people who assisted in harvesting the worms. We are especially grateful to Riekje Bruinenberg for the identification of the earthworms, Joachim Deru for the data analyses, and Maaike van Agtmaal for her comments and advice on the manuscript. The project was financed by the Project Lumbricus and the Public-Private Cooperation KLIMAP.

8. Supplementary data



Supplementary Figure S4.1 Penetration resistance of the soil at locations A and B.



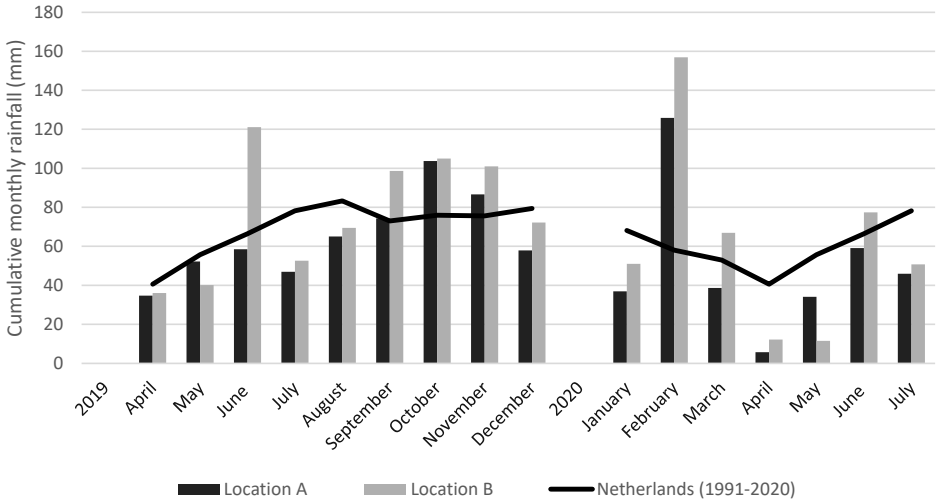
Supplementary Figure S4.2 Overview of the field experiment at location A.

Supplementary Table S4.1 Soil temperature (in °C) at 5, 10, 20, 50 and 100 cm depth at weather station Marknesse, located 50 km northwest of location A. The temperature was measured daily, four times per 24 hours (6:00, 12:00, 18:00 and 00:00); the temperature in the table is the average of all measurements in that month (https://www.knmi.nl/nederland-nu/klimatologie/bodemtemperaturen [*KNMI - Bodemtemperaturen*, n.d.]).

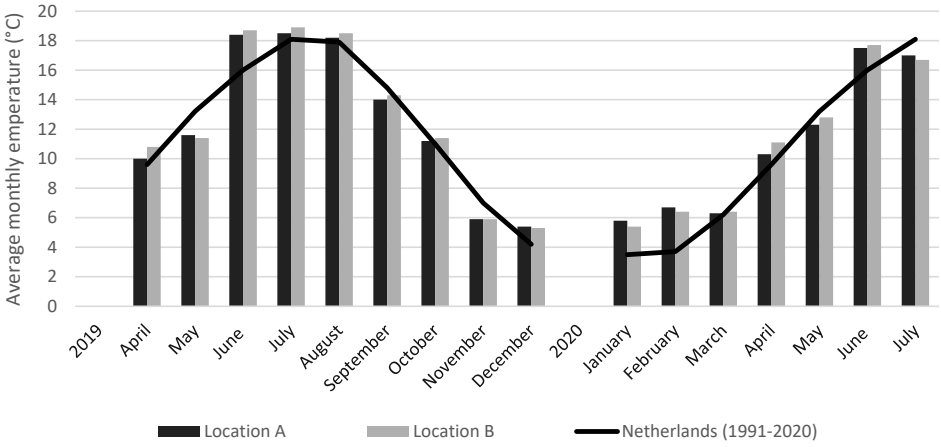
Marknesse	5 cm	10 cm	20 cm	50 cm	100 cm
Apr-19	9.8	9.7	9.6	9.4	9.0
May-19	12.9	12.7	12.6	12.1	11.1
Jun-19	18.7	18.0	17.7	16.6	14.5
Jul-19	19.3	19.0	18.9	18.2	16.7
Aug-19	19.1	19.0	18.9	18.5	17.4
Sep-19	15.4	15.8	15.9	16.3	16.3
Oct-19	11.8	12.4	12.6	13.4	14.0
Nov-19	6.5	7.2	7.5	8.7	10.5
Dec-19	5.0	5.5	5.7	6.6	8.0
Jan-20	5.5	5.6	5.7	6.2	7.2
Feb-20	6.0	6.2	6.3	6.6	7.2
Mar-20	6.4	6.7	6.7	7.0	7.5
Apr-20	10.9	10.5	10.2	9.7	9.1
May-20	14.4	13.9	13.6	12.8	11.6
Jun-20	18.8	18.3	17.9	16.7	14.6
Jul-20	18.7	18.6	18.5	17.9	16.5

Supplementary Table S4.2 Soil temperature (in °C) at 5, 10, 20, 50 and 100 cm depth at weather station De Bilt, located 30 km west of location B. The temperature was measured daily, four times per 24 hours (6:00, 12:00, 18:00 and 00:00); the temperature in the table is the average of all measurements in that month (https://www.knmi.nl/nederland-nu/klimatologie/bodemtemperaturen [*KNMI - Bodemtemperaturen*, n.d.]).

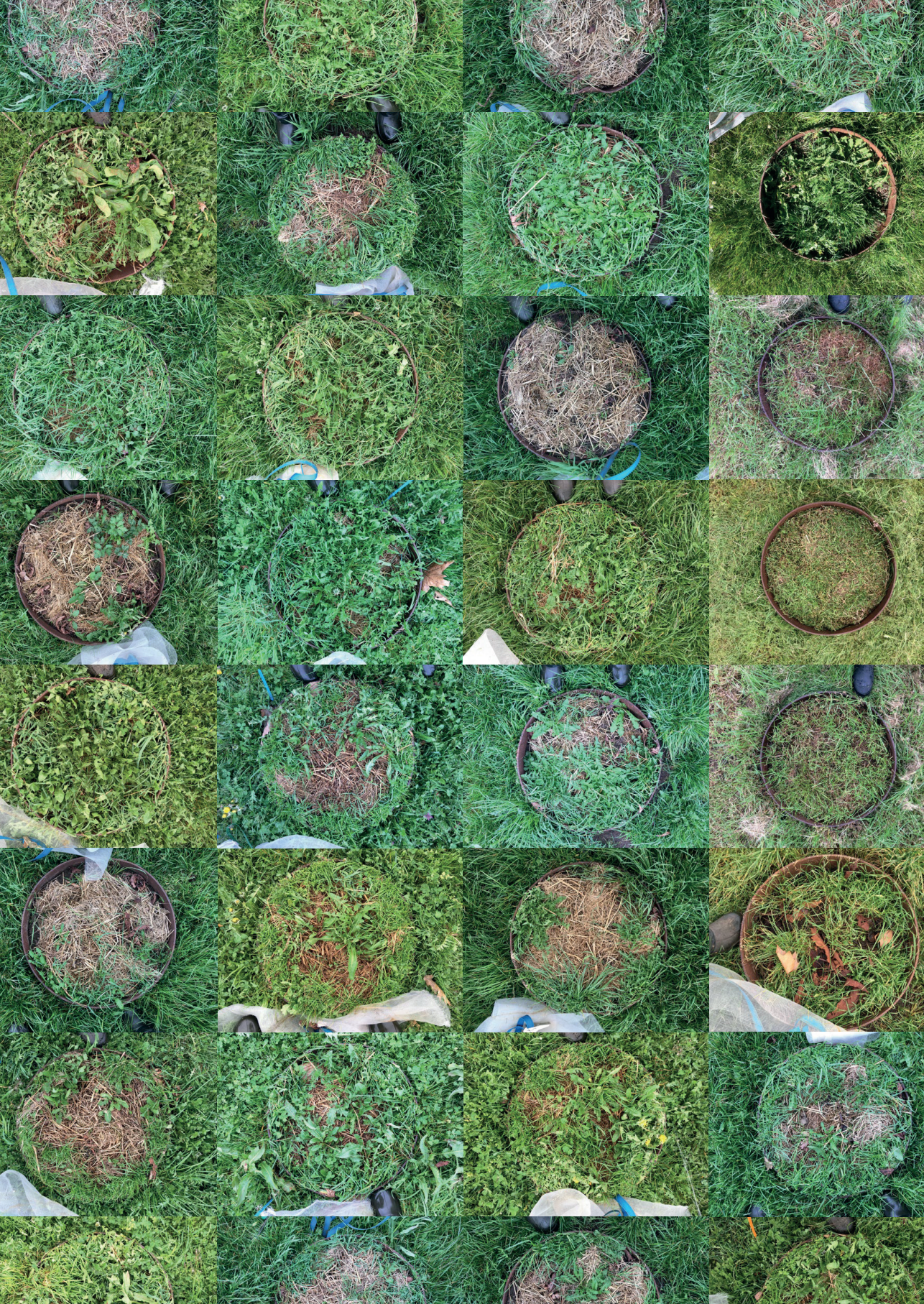
De Bilt	5 cm	10 cm	20 cm	50 cm	100 cm
Apr-19	10.5	10.3	10.1	9.7	9.3
May-19	12.8	12.6	12.2	11.6	11.0
Jun-19	17.3	17.0	16.4	15.2	13.9
Jul-19	18.9	18.8	18.2	17.2	16.1
Aug-19	18.5	18.5	18.2	17.7	16.9
Sep-19	15.9	16.1	16.1	16.3	16.2
Oct-19	13.2	13.5	13.6	14.3	14.6
Nov-19	8.4	8.7	9.1	10.3	11.5
Dec-19	6.5	6.7	7.0	8.0	9.1
Jan-20	6.4	6.5	6.7	7.4	8.2
Feb-20	6.8	6.9	7.0	7.5	8.0
Mar-20	7.0	7.2	7.2	7.6	7.9
Apr-20	10.3	10.3	9.9	9.5	9.1
May-20	13.9	13.8	13.1	12.2	11.4
Jun-20	17.8	17.5	16.7	15.3	14.1
Jul-20	17.8	17.7	17.3	16.5	15.6



Supplementary Figure S4.3 Cumulative monthly rainfall for locations A and B. Data retrieved from https://weerdata.nl (*Neerslag Statistieken per Plaats in Nederland*, n.d.) (based on data from KNMI weather station Heino for location A, and De Bilt for location B).



Supplementary Figure S4.4 Average air temperature (in °C) per month for locations A and B. Average temperature data retrieved from https://weerstatistieken.nl/ (*Neerslag Statistieken per Plaats in Nederland*, n.d.) (based on data from KNMI weather station Heino for location A, and Deelen for location B).



Chapter 5

Factors affecting colonisation success of the anecic earthworm *Lumbricus terrestris* (L.) in mesocosms on temperate pasture

Roos van de Logt and Nick van Eekeren

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Abstract

Background & aims: Changing precipitation patterns require climate adaptive measures to improve water regulation. Deep vertical earthworm burrows dug by anecic species *Lumbricus terrestris* (Linnaeus) contribute to water infiltration rate and capacity, and deeper root growth. *L. terrestris* is considered a native species to western Europe, reaching its highest abundances in pastures. In pastures where the species is currently absent, water regulation could improve after inoculation with these earthworms. We conducted a field experiment to test the feasibility of introducing *L. terrestris*.

Methods: Mesocosms were installed at two Dutch dairy farms. One farm had a resident *L. terrestris* population, the other did not. Subsequently, *L. terrestris* was introduced: half of the mesocosms received locally collected earthworms (NL), and the other half received commercially imported inoculum from Canada (CA). Twelve months later, the mesocosms were harvested and all earthworms were counted.

Results: The field experiment proved that *L. terrestris* can survive and produce offspring after introduction. At the location with a resident population, 15% of the *L. terrestris* introduced (tagged with Visible Implant Elastomer-tags) had survived, and at the *L. terrestris*-free site this was 26%. A supposed interspecific competitive relationship with *Lumbricus rubellus* (Hoffmeister) was not confirmed. Locally collected inoculum performed equal to or better than the commercial inoculum.

Conclusions: Threats to *L. terrestris* survival during the experiment could have been pathogens, competition, VIE-tag implantation and life history. Future research should focus on the role of pathogens and conducting this research in a non-enclosed set-up for a longer period of time.

Keywords: *L. terrestris*, anecic earthworms, pasture, water regulation, climate adaptation, ecosystem service

1. Introduction

The challenge of practising agriculture in a changing climate calls for climate adaptative strategies (Anderson et al., 2020). Ecological innovations can help to answer this call, for example with water regulation. Precipitation patterns in Europe have shifted, resulting in increased incidence of peak rains (Santos et al., 2016), combined with prolonged periods of drought (Samaniego et al., 2018; Grillakis, 2019). This shift can have a major impact on grasslands (Fitton et al., 2019), which represent one of the most prevalent land-use types of the continent. Agricultural grasslands cover over 48.0 million ha in Europe (*Eurostat 2023 - Cropping Patterns*) and play an important role in both food production and water regulation. Quick water infiltration is key to preventing flooding, runoff and associated nutrient loss during peak rains, and also to replenishing groundwater for more resilience in periods of drought. Deep rooting makes grasslands and arable crops more resilient to prolonged periods of drought.

The anecic earthworm *Lumbricus terrestris* (Linnaeus, 1758) occurs in grasslands, and it can address all of the aforementioned issues (Edwards et al., 1990; Bouché & Al-Addan, 1997; Spurgeon et al., 2013; Hallam & Hodson, 2020). This species creates semi-permanent (Potvin & Lilleskov, 2017) vertical burrows that can reach depths of up to two meters (C. A. Edwards & Bohlen, 1996). These burrows connect the soil surface to deeper soil layers, allowing water to flow through, while also facilitating plants to extend their roots further down (C. A. Edwards & Lofty, 1978). These features have been shown to improve the water infiltration rate and water infiltration capacity of grasslands (W. M. Edwards et al., 1990; Bouché & Al-Addan, 1997; Spurgeon et al., 2013; Hallam & Hodson, 2020). Introduction of *L. terrestris* into grasslands where it is absent could be an ecological method to improve the grassland's ecosystem service of water regulation.

In the Netherlands, 24% of the surface area is used as agricultural grassland (CBS website, 2024), mainly associated with dairy production. *L. terrestris* is considered a native species, and it occurs throughout the country and in the three major soil types in the Netherlands (clay, peat and sand). However, at the level of individual grasslands, its occurrence and abundance are heterogenous and this diversity cannot be easily explained. Field inventories on grasslands reported population densities varying from as low as 0 ind. m^{-2} to over 400 ind. m^{-2} (Van Eekeren et al., 2014; Van de Logt & Van der Sluijs et al., 2023).

Low abundance or even absence of *L. terrestris* does not necessarily mean that the grassland in question is unsuitable to accommodate the species. Previous research reports slow dispersal, with dispersion rates of 1.1 m $year^{-1}$ (Nuutinen et al., 2006), 1.4 m $year^{-1}$ (Ligthart & Peek, 1997), 4.5 m $year^{-1}$ (Hoogerkamp et al., 1983) and 6 m $year^{-1}$ (Shuster et al., 2003). Moreover, a fragmented landscape where individual farms or grasslands are surrounded by roads and waterways can make it impossible for *L. terrestris* to move from one grassland to the next. In combination with the low dispersal rate, this fragmentation may simply prevent the species from reaching new territory. Soil disturbance in the form of tillage can be detrimental to anecic earthworms such as *L. terrestris* (Chan, 2001; Van Eekeren et al., 2008; Fox et al., 2017) and could lead to local decimation or extinction of the population. Given the above, re-establishment can be difficult. Other specific factors may also have an effect on *L. terrestris* abundance. Correlative analysis of *L. terrestris* occurrence on sandy soil in relation to environmental factors indicated that the species is found more often and more abundantly in grasslands with a finer soil texture (Lindahl et al., 2009), i.e. loamier sand. *L. terrestris* was found less in grasslands with a high groundwater level, probably to avoid the risk of flooding of its burrows (Hoogerkamp et al., 1983; Van de Logt & Van der Sluijs et al., 2023). Additionally, interspecific competition for food with *Lumbricus rubellus* may play a role (Lowe & Butt, 2002; Shuster et al., 2003b; Van de Logt et al., 2023). Partial overlap in the dietary niche of the two species in combination with the higher metabolic rate and reproduction rate of *L. rubellus* may limit population growth of *L. terrestris*.

The *L. terrestris* invasion in Canada (Addison, 2009) and successful settlement of *L. terrestris* in the Dutch Flevopolder (Hoogerkamp et al., 1983) show that under suitable conditions, the species is able to disperse and establish itself. We expect that many grasslands have the potential to host *L. terrestris*, and the presence of *L. terrestris* is likely to improve the water-regulating capacity of these grasslands. Several research projects have explored the possibility of *L. terrestris* inoculation. Insights have been gained into *L. terrestris* survival rate (Butt, 1999; Andriuzzi et al., 2015; Forey et al., 2018), offspring production (Van de Logt et al., 2023), dispersal (Hoogerkamp et al., 1983; Ligthart & Peek, 1997), life history (Grigoropoulou & Butt, 2010), the effect of management (Grigoropoulou & Butt, 2010; Nuutinen et al., 2011; Kautz et al., 2014; Frazão et al., 2019) and experimental set-up (Butt, 2008). It was shown that *L. terrestris* can survive for more than one year and also produce offspring after being introduced in a grassland mesocosm set-up (Van de Logt et al., 2023). Despite

the potential of inoculation, so far the survival and reproduction rate on sandy soils have not been high enough to conclude that *L. terrestris* inoculation of grasslands is a viable ecological innovation that can contribute to climate adaptation through improved water regulation. In this research we aim to increase our understanding of which factors determine colonisation success of *L. terrestris*.

Building upon the work of previous research, we designed and executed an *L. terrestris* inoculation experiment in mesocosms on two locations: unique about this set-up was that one location was selected where *L. terrestris* was already present and one where this species was absent. Also, both locally collected and commercially purchased earthworms were used, as to test for the effect of origin on colonisation success. The objective of the experiment was to test the following hypotheses: locally collected *L. terrestris* are better adapted to local field conditions and will therefore outperform purchased Canadian *L. terrestris*, resulting in (I) higher survival rate and (II) higher reproductive output. Moreover, we hypothesise that *L. terrestris* will benefit from the absence of a competing resident earthworm population, resulting in (III) higher survival rate and (IV) higher reproductive output in the mesocosm with hand-sorted soil. In particular, we expect to find (V) a negative correlation between *L. terrestris* and *L. rubellus* abundance.

2. Materials and methods

2.1 Study area

In September 2021, a field experiment was set up in the south of the Netherlands. We selected two permanent grasslands on sandy soil at two organic dairy farms which were 8 km apart. Location A was situated at 51°35'56.6"N 5°07'03.7"E and Location B at 51°38'31.4"N 5°12'25.3"E. Location A was selected because *L. terrestris* was already present and Location B was selected because *L. terrestris* was absent, as a baseline assessment indicated in the previous year. The baseline assessment was carried out following the same procedure at both farms: two 40*40*20 cm pits were dug at least 25 m apart and at least 10 m from the edge of the grassland. All soil from the pits was hand-sorted for earthworms and four litres of 0.01% allyl-isothiocyanate (AITC) solution was applied to the bottom of the pit to collect earthworms from deeper soil layers. All earthworms emerging at the pit surface within 20 minutes were collected, rinsed with water and stored in 70% ethanol for later species determination (see paragraph 2.6 for details). Soil samples (0-10 cm and 30-40 cm depth) were taken in

duplo and analysed by Eurofins (Table 5.1). The soil type at Location A can be classified as Typic Humaquept, with a higher silt content and groundwater table (Table 5.1) than Location B, which can be classified as Typic Haploquod (USDA classification). Both grasslands were dominated by perennial rye-grass (*Lolium perenne*) and white clover (*Trifolium repens*), management consisted of cutting and grazing at both farms (Table 5.1) with a total N-input of 170 kg N ha^{-1} from slurry manure, as well as from dung and urine from grazing. Because of its soil texture and groundwater table, Location B was more sensitive to drought and was irrigated during prolonged periods of drought (see Suppl. Figure S5.2 for weather data and Suppl. Table S5.2 for soil temperature).

Table 5.1 Soil analysis and management of Locations A and B. Each value represents the average of the two samples that were taken

Site	Location A	Location B
Chemical properties 0-10		
pH-KCl	5.3	6.0
Organic matter (%)	5.2	4.4
N-total (g N/kg dry soil)	2.21	2.02
P-Al (mg pP_2O_5/100 g)	63	71
% sand	65	81
% silt	27.5	13
% lutum	2.5	1.5
Chemical properties 30-40		
pH-KCl	5.2	5.6
Organic matter (%)	3.7	2.8
N-total (g N/kg dry soil)	1.41	1.12
P-Al (mg pP_2O_5/100 g)	58	75
% sand	67	83
% silt	28	13
% lutum	2	1
Groundwater stage	VIId	VIIIo
Management		
Cutting	Yes	Yes
Grazing	Yes	Yes
Irrigation	No	Yes
Fertilisation (total N kg ha^{-1})	170	170

2.2 Installation of the mesocosm field experiment

We set out the plots and installed the mesocosms in both grasslands. With a crane, steel pipes (ø 61 cm, height 50 cm) were driven 40 cm into the soil, leaving a 10 cm ring above the soil surface (Suppl. Figure S5.1). Pipes were installed in two rows of equal length, and pipes were spaced 90 cm apart. We assigned the mesocosms to six different treatments in a randomised block design (Table 5.2). Four blocks were installed at Location A and three blocks were installed at Location B.

Table 5.2 Overview of treatments

Treatment	Soil	Earthworms
1. Undisturbed soil, control	Undisturbed	No earthworm inoculation
2. Disturbed soil, control	Hand-sorted and returned to mesocosm	Resident population removed
3. Undisturbed soil, Canadian *L. terrestris (CA)*	Undisturbed	Inoculated with 15 commercially purchased *L. terrestris* (51 m^{-1})
4. Disturbed soil, Canadian *L. terrestris (CA)*	Hand-sorted and returned to mesocosm	Resident population removed. Inoculated with 15 commercially purchased *L. terrestris* (51 m^{-1})
5. Undisturbed soil, Dutch *L. terrestris (NL)*	Undisturbed	Inoculated with 15 locally collected *L. terrestris* (51 m^{-1})
6. Disturbed soil, Dutch *L. terrestris (NL)*	Hand-sorted and returned to mesocosm	Resident population removed. Inoculated with 15 locally collected *L. terrestris* (51 m^{-1})

At the time of installation, from half of the mesocosms (treatments 2, 4 and 6), the soil was removed in three layers: 0-20 cm, 20-40 cm, and 40-60 cm. The soil removed was hand-sorted on site to remove all earthworms (cocoons were left in the soil). No *L. terrestris* was found at Location B, re-confirming the absence of the species from this grassland. After hand-sorting, the soil was returned to the mesocosm it had been removed from, with each layer in its original place, and subsequently grass-clover was seeded. The mesocosms under this treatment are referred to as 'disturbed soil'.

2.3 Earthworm origin

The earthworms used as inoculum had two different origins. The first group of *L. terrestris* earthworms for the experiment was purchased commercially from a Dutch earthworm importer (https://www.thedutchnightcrawlers.nl/). The earthworms purchased originated from Ontario, Canada, where they had been collected from agricultural fields to serve the bait market (for more details, see Steckley, 2020). These

earthworms are also referred to as 'CA' inoculum. The second group of *L. terrestris* was collected at Location A through mustard extraction. A rectangular, 15 cm deep pit was dug, and to this pit a mixture of water and mustard powder was applied (50 gr of Coleman's mustard powder per 10 L of water, based on recommendations of Dr K. Butt (personal communication). Earthworms that surfaced in the following 45 minutes were carefully collected, without pulling them, and immediately rinsed with tap water. Until the moment of introduction, the earthworms collected were kept in a bucket with soil, grass-clover cuttings and cow manure from the resident grassland. These earthworms are referred to as 'NL inoculum'.

To enable distinguishing the introduced *L. terrestris* from resident *L. terrestris* after harvesting the experiment, we tagged earthworms destined for inoculation at Location A. Visible Implant Elastomer (VIE) tags were prepared following the instructions from the supplier (Northwest Marine Technology, Inc.). A droplet of elastomer was injected behind the clitellum of all *L. terrestris* that were to be introduced at Location A (Butt & Lowe, 2007).

2.4 Earthworm inoculation

Twenty holes (ø 0.4 cm, 5 cm in depth) were made in the soil surface of all mesocosms. These holes served as 'easy access points' for the *L. terrestris* that were to be introduced; for consistency, they were also made in the control mesocosms. Fifteen *L. terrestris* earthworms (51 m^{-2}) were introduced into the assigned mesocosms. Half of these mesocosms received CA *L. terrestris* (treatments 3 and 4) and half of the mesocosms received NL *L. terrestris* (treatments 5 and 6). One bucket-full of autumn leaves (*Aesculus hippocastanum*) was spread out on the soil surface of each inoculated mesocosm before the surface was covered with mesh gauze. Control mesocosms also received one bucket-full of autumn leaves each, and then they too were covered with 1.5 mm mesh gauze to ensure that no earthworms would enter or leave the mesocosm, neither by themselves nor through predation. A fence was put up around the experimental plot to prevent cows from entering and reaching the mesocosms.

2.5 Maintenance of the field experiment

The field experiment was checked regularly and in January 2022, 800 grams of farmyard manure from Location B was applied to each mesocosm by spreading it evenly over the soil surface. The fertilising value of the manure was analysed by Eurofins, Wageningen (Suppl. Table S5.1).

In April 2022, grass-clover was seeded again in the mesocosms with disturbed soil, as germination had been poor. From April onwards, the vegetation was cut regularly, both inside the mesocosms and in the rest of the plot. All mesocosms were provided with cuttings to ensure food supply for the earthworms. In May, July and August, the farmer at Location B irrigated the grassland on which the experiment was situated. A total of four irrigations took place, 22 mm each time. In May and August, we watered the mesocosms at Location A with ~18 mm, as the soil was becoming very dry in the absence of rain. More detailed information about the weather conditions during the course of the experiment (temperature, rainfall and soil temperature) can be found in Suppl. Table S5.2.

2.6 Mesocosm harvest

In September 2022, all mesocosms were harvested in the same way. The soil was removed from each mesocosm in three layers (0-20 cm, 20-40 cm, 40-60 cm), using a spit fork to minimise the risk of damaging the earthworms. Each soil layer was hand-sorted to collect all the earthworms that it contained. Mustard extraction was used to also collect earthworms from deeper soil layers. Six L of water with mustard powder (see paragraph "*Earthworm origin*" for more details) was applied to the pit of each mesocosm (60 cm depth). The bottom of the pit was checked regularly for at least 30 minutes, and all earthworms that emerged were rinsed with tap water and processed like the rest of the earthworms. Earthworms were kept cool and taken to the laboratory, and the following day they were rinsed, weighed and put in 70% ethanol to store them until species determination. Hand-sorted soil was returned to the same mesocosm from which it had been taken, and all three layers were put back in their original order.

2.7 Species determination

Earthworms were identified to species level when possible. Individuals were categorised as adult if a clitellum was present or emerging, and they were categorised as juvenile if a clitellum was completely absent. Juveniles that belonged to either *L. rubellus* or *L. terrestris* were distinguished from each other by checking for considerable thickenings of any septa, which, as described by Stöp-Bowitz (1969), are absent for *L. rubellus*. No other species that could have been interchanged with *L. rubellus* or *L. terrestris* were present in the samples.

2.8 Statistical analysis

Prior to analysis, the collected data was checked for normality and transformed when needed. Rstudio version 2023.12.0+369 software (R Core Team, 2023) was used to perform linear regression analyses and ANOVA. Factors were *location* (A and B), *inoculum* (either locally collected inoculum [NL] or imported Canadian specimens [CA]; this was not applicable for Control [C] mesocosms as these were not inoculated), and *soil disturbance* (undisturbed [U] and disturbed [D]). LSDs were used for the interpretation of two- and three-way interactions. Excel (Version 2409 Build 16.0.18025.20160) was used for a two-sample t-test on data about tagged earthworms.

3. Results

3.1 Effects of location, soil disturbance and inoculation on *L. terrestris* abundance

Location had a significant effect on total *L. terrestris* abundance, adult *L. terrestris* abundance and juvenile *L. terrestris* abundance (Table 5.3). For this reason, the results are described separately per location in the following paragraphs. Inoculation of the mesocosms resulted in higher total *L. terrestris* abundance and higher adult *L. terrestris* abundance, but it did not change juvenile *L. terrestris* abundance (Table 5.3).

3.2 *L. terrestris* survival and offspring at Location A

At Location A, where *L. terrestris* already occurred, the introduction of *L. terrestris* did not lead to increased *L. terrestris* abundance, neither in the plots with undisturbed soil nor in the plots with disturbed soil from which the resident earthworm population had been removed. Neither the adult nor juvenile fraction of the *L. terrestris* population was affected by inoculation treatment (Figure 5.1A). A total of 37 tagged earthworms was retrieved from inoculated mesocosms, which amounts to 15% of the original inoculum (Table 5.4 and Figure 5.1A). There was a tendency towards higher NL than CA *L. terrestris* retrieval ($p = 0.076$) among tagged specimens. The difference in the number of *L. terrestris* retrieved from disturbed and undisturbed mesocosms was not significant (Figure 5.1A). At the level of each individual mesocosm (both inoculated and control), the number of juveniles per adult was variable and ranged from 1.5 to 13.7, with an average of 4.93.

Table 5.3 Overview of earthworm biomass and abundance per location, soil disturbance category, inoculation type and interactions between these. Significance is indicated as *, ** and *** when p-value was < 0.05, < 0.01 and < 0.001 respectively; non-significant p-values are indicated with "ns"; p-values < 0.05 > 0.1 are considered tendencies, these are indicated as p-values. 'U' is 'undisturbed soil', 'D' is disturbed soil from which the resident earthworm population was removed before the field experiment set off, 'CA' refers to inoculation with Canadian *L. terrestris*, 'NL' refers to inoculation with locally collected *L. terrestris*, 'C' refers to control treatment. When data was transformed to obtain normal distribution this is indicated. NB: only at Location A earthworms were tagged, na = non applicable.

		Means							p-value							
		Location (L)		Soil disturb. (S)		Inoculum (I)			Main effects			Interaction effects				
	Unit	A	B	U	D	CA	NL	C	L	S	I	L*S	L*I	S*I	L*S*I	Trans.
Tot. biomass	gr	158	164	142	179	159	171	153	ns	.057	ns	ns	ns	ns	ns	-
Tot. earthworms	#	550	345	405	519	457	477	453	*	.077	ns	ns	ns	ns	ns	-
Tot. epigeics	#	34	147	77	87	57	101	87	***	ns	**	ns	*	*	ns	-
Tot. endogeics	#	385	185	246	353	323	286	290	*	*	ns	ns	ns	ns	ns	sqrt
Tot. anecics	#	132	13	82	80	77	90	76	***	ns	*	ns	**	ns	ns	sqrt
Tot. *L. terrestris*	#	128	13	81	77	76	87	73	***	ns	*	ns	.085	ns	ns	sqrt
Ad. *L. terrestris*	#	23	9.0	16	18	16	24	12	***	ns	*	ns	ns	ns	ns	-
Juv. *L. terrestris*	#	105	4.0	64	59	60	63	62	***	ns	ns	ns	ns	ns	ns	sqrt
Tagged *L. terrestris*	#	7.9	na	5.6	11.0	5.1	10.7	n.a.	na	ns	0.076	na	na	na	na	-
Tot. *L. rubellus*	#	22	124	65	67	44	87	67	***	ns	*	ns	.080	ns	ns	-
Ad. *L. rubellus*	#	0.7	47	18	24	17	19	25	***	ns	ns	ns	.080	ns	.053	sqrt
Juv. *L. rubellus*	#	21	77	47	43	27	67	41	***	ns	**	ns	ns	ns	ns	-

Table 5.4 Number of VIE-tagged *Lumbricus terrestris* specimens recovered, per treatment at Location A

Treatment	Number of retrieved tagged *L. terrestris*	Number of untagged *L. terrestris*	% tagged
Undisturbed soil, NL	8	22	27
Undisturbed soil, CA	5	20	20
Disturbed soil, NL	17	27	39
Disturbed soil, CA	7	11	39
Total	37	80	32

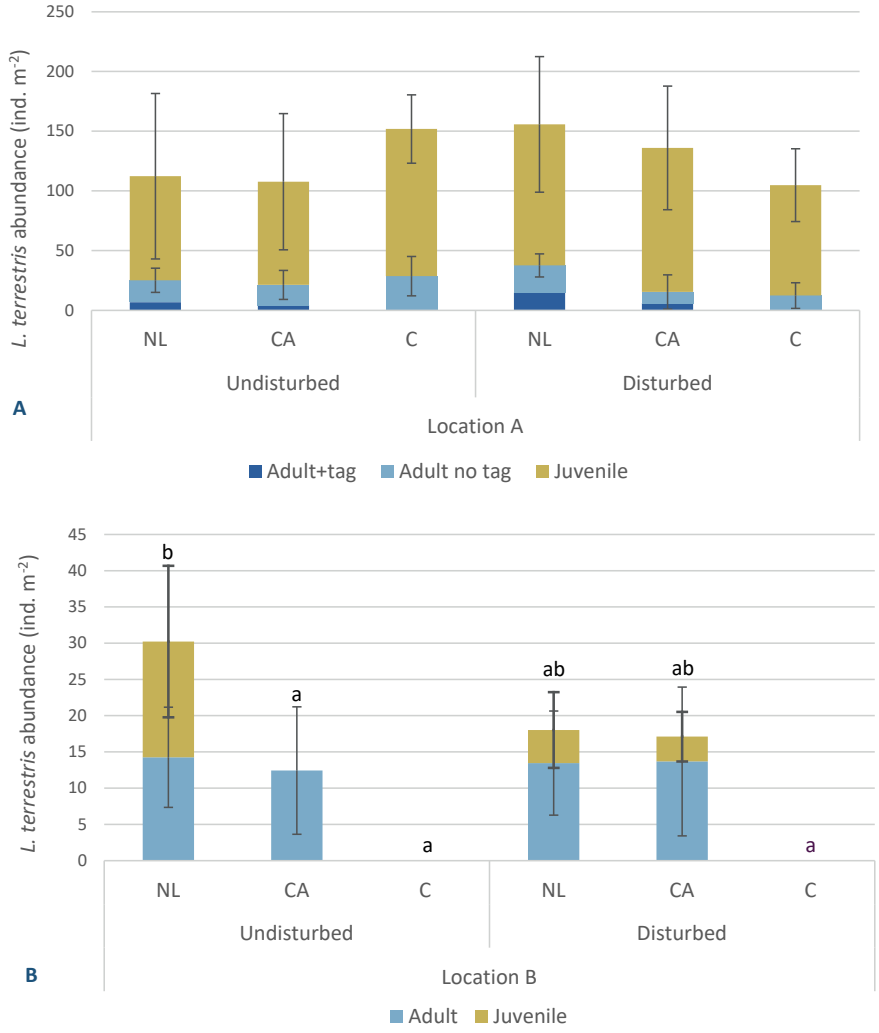

Figure 5.1A and 5.1B Adult (tagged: dark blue, untagged: light blue) and juvenile *Lumbricus terrestris* per treatment and soil disturbance category for Location A (resident *L. terrestris* population present) and Location B (no resident *L. terrestris* population present). Standard error bars refer to juveniles and adults separately; tagged adults and untagged adults are combined in one group.

3.3 *L. terrestris* survival and offspring at Location B

At Location B, where *L. terrestris* did not occur before inoculation, *L. terrestris* introduction resulted in a significantly higher *L. terrestris* abundance of both adults and juveniles, compared to controls (Figure 5.1B). Overall, 26% of the *L. terrestris* introduced survived twelve months in the mesocosms, and they produced a mean of 0.44 juveniles per adult. Survival rates were comparable for NL and CA inoculum (27% and 25%, respectively) The highest mean survival rate – although non-significant – occurred in undisturbed mesocosms with NL inoculum. This treatment yielded a mean population density of 30 *L. terrestris* m^{-2} (51 ind. m^{-1} had been introduced), with equal numbers of adults and juveniles (Figure 5.1B).

At Location B, soil disturbance and the removal of earthworms did not have a significant effect on total and adult *L. terrestris* abundance in inoculated mesocosms (Figure 5.1B). Among the juvenile fraction, there was a significant interaction effect between soil disturbance and inoculation treatment. In undisturbed mesocosms, NL exceeded CA in juvenile abundance (3.85 m^{-2} and 0 m^{-2}, respectively), whereas in disturbed mesocosms there was no such difference between NL and CA (Figure 5.1B). At the level of each individual mesocosm, the number of juveniles per adult varied between 0.0 and 4.0 (average of 0.7).

3.4 Relation between *L. terrestris* and resident earthworm population in general

At Location A, seven different earthworm species were found whose average abundance was 550 ind. m^{-2} (Suppl. Table S5.3). All three major ecological categories (epigeic, endogeic, anecic) were represented. Numerically, *Aporrectodea caliginosa* was the most dominant species, followed by *L. terrestris*; together they amounted to 70% of the total earthworm population. The juvenile fraction of the earthworm population was 0.68. At Location B, five different earthworm species were found, among which no anecic species, apart from the introduced *L. terrestris*. The average earthworm population density was 345 ind. m^{-2}. Equal to Location A, *A. caliginosa* was the most abundant species, and *L. rubellus* was the second most common species; together these two amounted to 86% of all earthworms. The juvenile fraction of the earthworm population was 0.51.

Among the resident earthworm species, four species significantly correlated to *L. terrestris* in abundance (Table 5.5). *L. rubellus* and *A. chlorotica* showed a significant positive correlation at Location B but not at Location A. *L. castaneus* was only present at Location B, where its abundance correlated significantly positively to that of *L.*

terrestris. *A. nocturna* was only found at Location A, where its abundance correlated positively to that of *L. terrestris*.

Table 5.5 Pearson's r correlation coefficient, R^2 and corresponding p-values for correlations between the abundance of *L. terrestris* and co-occurring species. For Location B, only inoculated mesocosms were included. na = not applicable (due to absence of species), ns = non-significant, *, ** and *** refer to significance levels of > 0.05, > 0.01 and > 0.001, respectively.

Species	Location	Pearson's r	R^2	p-value
L.ter x L.cas	A	na	na	na
	B	0.144	0.654	*
L.ter x L.rub	A	-0.085	0.005	NS
	B	0.755	0.694	**
L.ter x A.ros	A	-0.445	0.029	ns
	B	na	na	na
L.ter x A.chl	A	0.522	0.009	NS
	B	0.013	0.969	***
L.ter x A.cal	A	0.679	0.000	ns
	B	0.579	0.049	ns
L.ter x A.lim	A	0.563	0.004	ns
	B	na	na	na
L.ter x A.noc	A	0.134	0.503	*
	B	na	na	na

3.5 Interaction between *L. terrestris* and *L. rubellus*

Considering both locations together, total *L. rubellus* abundance was lowest in the CA treatment. Highest *L. rubellus* juvenile abundance was found for the treatment of NL inoculation. These differences can be ascribed to the large juvenile *L. rubellus* fraction, as the adult fraction did not yield any significant relation to inoculum treatment. Control mesocosms hosted an intermediate *L. rubellus* abundance, which did not differ significantly from either the NL treatment or the CA treatment (Table 5.3).

The abundance and demography of *L. rubellus* differed between the two locations. At Location A, there were hardly any adult *L. rubellus* (1%), whereas at Location B, each mesocosm contained both adults (34%) and juveniles (66%) (Figure 5.3). At Location A, the population density of *L. rubellus* varied strongly per mesocosm, ranging from 0 to 79 ind. m^{-2}; no correlation was found between the abundances of *L. rubellus* and *L. terrestris* (Pearson's r = -0.085; R^2 = 0.007). A positive correlation between the number of *L. terrestris* and *L. rubellus* was found at Location B (Pearsons's r = 0.755; R^2 = 0.570) (Figure 5.2). Here, the population density of *L. rubellus* ranged from 17 to 232 ind. m^{-2}.

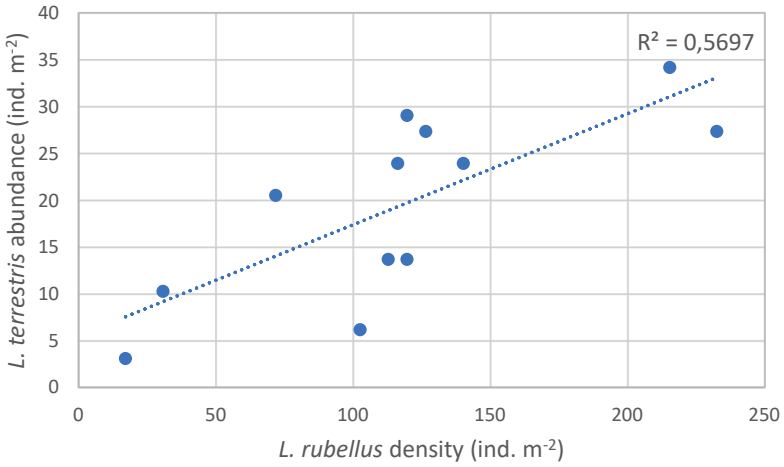

Figure 5.2 Correlation between *Lumbricus rubellus* abundance and *Lumbricus terrestris* abundance at Location B.

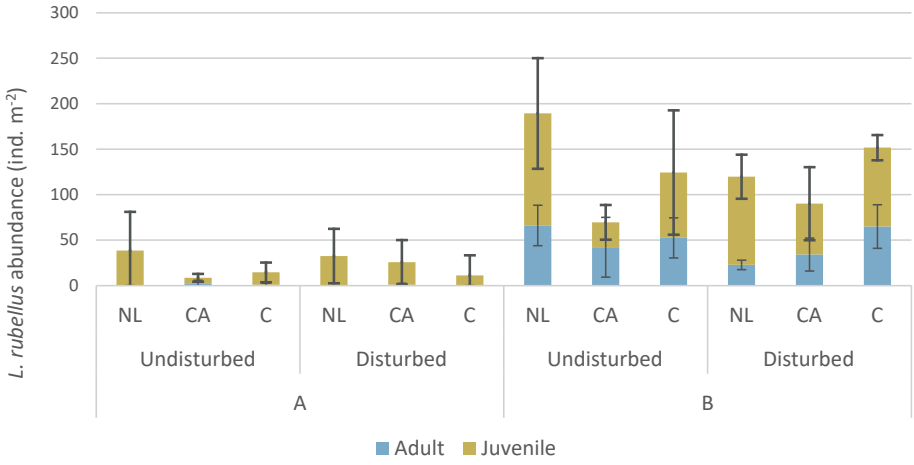

Figure 5.3 Adult and juvenile *Lumbricus rubellus* abundance (ind. m^{-2}) per treatment, soil disturbance and location, no significant differences in either location. Standard error bars refer to juveniles (in bold) and adults (normal) separately.

4. Discussion

4.1 General *L. terrestris* survival and reproduction

At both locations and in all inoculation treatments, inoculated *L. terrestris* were retrieved after twelve months. At Locations A and B, the recovery rate of inoculated *L. terrestris* was 15% (tagged specimens) and 26%, respectively. At Location B, offspring was found in all treatments (0.44 juveniles per adult on average) except for the CA inoculum in undisturbed soil.

In previous field studies, the reported *L. terrestris* retrieval ranges from as low as 0% to as high as 80%. In a field trial with a similar set-up that we executed in 2019-2020, we recorded 33% retrieval after eight months, with 0.17 juvenile per adult, and 6% survival after fifteen months, with 0.90 juvenile per adult (Van de Logt et al., 2023). A mesocosm study on arable land by Andriuzzi et al. (2015) reported 40% retrieval from inoculated mesocosms after eight months. Also on arable land, Frazão et al. (2019) observed an estimated 14% recovery and 3.3 juveniles per adult after one year, but no recovery after two years. Forey et al. (2018) found at least 15% survival after 12 months on grassland, but as they did not sample deeper than 50 cm, it is possible that not all surviving earthworms were found. In a twelve-month field trial by Rajapaksha et al. (2014), mesocosms were inoculated with *L. terrestris* and 80% of the earthworms were recovered. Lab inoculation studies in a controlled environment resulted in *L. terrestris* survival rates of 68% (Butt, 1991) and 100% after one year (Butt et al., 1992), along with 45% survival after more than two years (Svendsen et al., 2005).

It is difficult to identify the cause of the high variability in survival rates among this and previous inoculation experiments. Andriuzzi et al. (2015) speculate that both mortality and inefficiency of the sampling method could explain limited retrieval. Forey et al. (2018) and Van de Logt et al. (2023) suspect drought to have been fatal to the earthworms. Van de Logt et al. (2023) also discuss the potentially detrimental effects of predation, of the life history of the *L. terrestris* introduced, and of competitive interaction with the resident earthworm population. In the set-up of our experiment, we aimed to rule out the risks of predation, drought, competition for food, unsuitable habitat and unfit life history (harvest, storage, transport process). To this end, we covered the mesocosms with nets, watered the plot in prolonged periods of drought, provided leaf litter, farmyard manure and grass cuttings, introduced *L. terrestris* into two different grasslands, one where the species already occurred (Location A) and one where it was absent (Location B), and introduced both commercially purchased *L. terrestris* from Canada (CA) and locally collected specimens (NL) for comparison. We first discuss our findings separately for each location (paragraph 4.2) and then conclude with remarks that apply to both (paragraphs 4.3 and 4.6).

4.2 *L. terrestris* survival and reproduction per location

4.2.1 Location A

At Location A, there were no significant differences in total *L. terrestris* abundance between the treatments. It was not feasible to assess offspring production of the *L. terrestris* introduced, as inherent to the set-up of the experiment, it was impossible to distinguish between the offspring of introduced *L. terrestris* and the offspring of resident *L. terrestris*.

Three remarkable observations that we made are discussed here. Firstly, we had expected that Location A would yield a higher survival rate than Location B, especially because the NL inoculum was introduced back into the same grassland that the earthworms had been collected from, and therefore we were certain to have introduced them into a suitable habitat. However, being removed, tagged, stored and returned to the grassland seems to have had a stronger negative effect on the earthworms at Location A than at Location B. We consider it unlikely that the difference can be ascribed to removal, storage or inoculation, as these procedures were identical for both locations. Tagging and the presence of resident *L. terrestris* are the most obvious differences between Locations A and B. It is possible that niche-saturation did not allow for an increase in *L. terrestris* population density. Potentially negative effects of VIE tagging are discussed in more detail in paragraph 4.2.2.

The second observation was that the inoculation of undisturbed plots with *L. terrestris* did not lead to increased population density compared to controls after one year, possibly due to niche-saturation at Location A. Given the feeding regime, we consider it unlikely that food was a limiting resource, but perhaps competition for space played a role. Unfortunately, we cannot separate this from the impact that VIE-tagging might have had.

The third observation was that the total numbers of *L. terrestris* in undisturbed and disturbed control mesocosms were more similar than expected. At the onset of the field trial, earthworms had been removed from these mesocosms by hand-sorting to a depth of 60 cm, combined with mustard extraction (see paragraph 2.3). It was not expected that this would lead to complete absence of *L. terrestris* from disturbed control mesocosms, as cocoons were not removed. However, it was supposed that the effects of manual removal and soil disturbance would be at least comparable to intensive cultivation, which has been demonstrated to be able to severely decimate

the *L. terrestris* population (Chan, 2001; Pelosi et al., 2014; Frazão et al., 2019). However, the populations in the disturbed control mesocosms appeared fully recovered after 12 months (Figure 5.1A). It is possible that the resource-rich and protective environment of the mesocosms and the loosened soil, combined with the removal of the vast majority of possible competitors, facilitated rapid population recovery from the cocoons and juveniles that had remained. What is interesting about this observation is that it suggests that under suitable conditions, population growth of *L. terrestris* can be rapid. This implicates that management interventions can contribute to stimulating the existing *L. terrestris* population in grasslands.

At Location A we retrieved a total of 37 from the 240 *L. terrestris* introduced (15%), which comprised 25 NL and 12 CA specimens. Among the *L. terrestris* retrieved, there were some interesting treatment effects (Table 5.3, Table 5.4). NL earthworms outperformed CA earthworms, matching our hypothesis that locally collected *L. terrestris* have a higher survival rate than commercially purchased inoculum. We think this indicates that locally collected *L. terrestris* are more readily adapted to the environment. Paragraph 4.2.2 elaborates on this. Another interesting difference was that, as we expected, more tagged *L. terrestris* were found in disturbed soil than in undisturbed soil. A possible explanation could be that intraspecific competition was very low at the moment of introduction, making it easy to settle. Additionally, the loosened soil likely facilitated easier burrowing, allowing the earthworms to allocate their resources to other processes important for survival. The absence of resident earthworms and loosened soil are confounding factors in this experimental set-up, making it impossible to distinguish between them.

Contrary to our hypothesis, no correlation between the abundance of *L. rubellus* and *L. terrestris* was found at Location A, whereas previous studies seemed to suggest a negative interaction between *L. rubellus* and *L. terrestris* (Shuster et al., 2003a; Van de Logt et al., 2023; Van de Logt & Van der Sluijs et al., 2023). Perhaps no correlation was found because the abundance of *L. rubellus* was low (on average 22 ind. m^{-2}) and almost completely represented by juveniles, making competition for resources between the species under the circumstances as they were during the experiment highly unlikely.

4.2.2 Location B

At Location B, a total of 26% of the inoculum (NL and CA) was retrieved, with no significant difference in *L. terrestris* abundance between disturbed and undisturbed mesocosms, nor between NL and CA inoculum treatments. Still, among the undisturbed mesocosms, NL *L. terrestris* outperformed CA *L. terrestris*, mainly because the latter failed to produce offspring. The general recovery rate was higher than at Location A, and it also exceeded the recovery rate that we found in our previous field trial (Van de Logt et al., 2023). As mentioned above, the experimental set-up of the present field trial was based on the set-up that we used in a previous study (Van de Logt et al., 2023), but with some adjustments (i.e. more irrigation, more food supply, and local inoculum). These adjustments may have improved the success of the introduction, and we will discuss them below.

First, more food was provided by leaving grass cuttings in the mesocosm instead of removing them. It was decided to do this as we considered resource availability a possible limiting factor in the previous study, mainly because of the seemingly negative interspecific interaction with *L. rubellus*, which feeds within the same niche. Contrary to previous findings (Van de Logt & Van der Sluijs et al., 2023; Van de Logt et al., 2023), we observed a positive correlation between *L. rubellus* and *L. terrestris* (Figure 5.2). Thus, these two species do not necessarily compete. The grass cuttings may have ensured ample resource availability and prevented interspecific competition. Second, the grassland that hosted the field experiment was irrigated several times during the summer; if drought had been a problem in the previous experiment as had been suggested, it is safe to assume that irrigation contributed to increased *L. terrestris* survival. Third, no damage to the covering nets was inflicted by birds, eliminating the concerns about predation that we had in our previous experiment.

At Location B, we found a mean of 0.44 juveniles per adult, which seems too few to rejuvenate the population. However, cocoons were not collected and hence not included. Collecting and identifying cocoons is laborious but not impossible; for future research we would suggest including cocoon data. Furthermore, *L. terrestris* population abundance and demography fluctuate throughout the year (Potvin & Lilleskov, 2017). Our harvest took place in September, after 12 months, but peaks in juvenile abundance are more likely to be found in November (Daniel, 1992). Lastly, the fact that the number of juveniles per adult ranged from 0.0 (undisturbed plots with CA inoculum) to 4.0 (undisturbed plots with NL inoculum) demonstrates that in some mesocosms the *L. terrestris* community showed potential for rejuvenation

and population growth. Long-term experiments are necessary to validate long-term population development.

4.3 Factors affecting *L. terrestris* persistence and survival

As is outlined by our results as well as by previous work by Rajapaksha et al. (2014), Andriuzzi et al. (2015), Forey et al. (2018), Frazão et al. (2019) and Van de Logt et al. (2023), survival rates are highly variable. The question arises whether these results and studies report on *L. terrestris* populations that are dying out, or on populations that are going through a phase which is eventually part of long-term settlement? In the latter case, part of the inoculum may be unfit for the new territory and thus fail to survive, whereas the remaining earthworms are able to thrive and reproduce, leaving the apt and their offspring to colonise the area in time to come. Unfortunately, the number of juveniles per adult is not mentioned in most studies (Rajapaksha et al., 2014; Andriuzzi et al., 2015; Forey et al., 2018;), probably due to difficulty in species determination for juveniles. However, species determination for juvenile *L. terrestris* is possible in many cases (see also paragraph 2.7). We think it is of vital importance to include juvenile abundance, since juveniles can make up a large part of the population (Figure 5.1) and offspring production is simply indispensable for the persistence of the population. Consequently, monitoring of juveniles is a necessity in assessing whether a population is moving towards extinction or has the potential to rejuvenate itself.

Below, we try to elucidate what factors may have threatened *L. terrestris* population persistence in our experiment.

4.4 Life history of earthworms introduced

The life history of *L. terrestris* may have a strong influence on its performance after inoculation. Andriuzzi et al. (2015), Forey et al. (2018), Frazão et al. (2019), and Van de Logt et al. (2023) all used commercially purchased *L. terrestris*, despite the remark by Grigoropoulou and Butt (2010) that persistence of locally collected *L. terrestris* was significantly greater than the persistence of commercially purchased specimens, probably because commercial purchase is cheap, easy and less labour-intensive. Rajapaksha et al. (2014) used field-collected earthworms.

In our previous paper we suggested that it seems reasonable to assume that locally-collected earthworms experience a smaller disruption of their ‘normal life’ and therefore have a better starting position at the moment of introduction (Van de Logt

et al., 2023). Perhaps because they did not go through the post-harvest process of transportation, storage, artificial feeding conditions, indoor climate conditions and long-distance shipping to the Netherlands as described by Steckley (2020). Apart from logistics, the change in physio-chemical and microbial environment from heavily manured alfalfa fields in Ontario, Canada, to organic grasslands on sandy soil in the Netherlands may have severely challenged the imported *L. terrestris* that we introduced. Analysis of the genetic population differentiation between *L. terrestris* from a German meadow and *L. terrestris* from a Canadian reference population resulted in a considerable difference of 15.17% (Velavan et al., 2009). Part of the potential for adaptation to the environment could be embedded within this genetic variation.

It appeared that the difference in persistence between NL and CA *L. terrestris* was not the same for Locations A and B. At Location A, the majority (68%) of the retrieved *L. terrestris* were NL specimens, suggesting a 'home-advantage' for locally collected earthworms. At Location B, there was no significant difference in survival rate between NL and CA earthworms (27% and 25%, respectively). We speculate that this may mean that habitat adaptation is very site-specific. In other words, for the inoculum, the difference between Location A and Location B was not smaller than the difference between their Canadian origin grounds and Location B; therefore, the 'homefield-advantage' of the NL *L. terrestris* was possibly lost and survival rates ended up similar. However, as our field experiment was small in scale and the survival rate at Location A was generally low, more research is required to validate this hypothesis.

4.5 Pathogen pressure

The highest *L. terrestris* recovery rate in a field study in literature was 80% after one year. This was found in a study conducted on sterilised soil (Rajapaksha et al., 2014). All other studies were carried out on unsterilised soil. The sterilisation procedure may have killed pathogens that otherwise would have been harmful to *L. terrestris*. In current literature, interactions between *L. terrestris* and soil-borne pathogens are very rarely addressed. The effect of parasites on overall survival, growth and fecundity of *L. terrestris* is addressed even less (Edwards & Arancon, 2022), neither from the perspective of earthworm introduction nor from earthworm invasion. However, *L. terrestris* interacts intimately with soil biota, as has been revealed by analyses of gut content, midden material and drilosphere (Tiunov & Dobrovolskaya, 2002; Nechitaylo et al., 2010; Stromberger et al., 2012). Therefore, it seems reasonable to assume that pathogenic soil biota could have a strong effect on *L. terrestris* survival. It is possible

that the inoculated *L. terrestris* (both NL and CA) in our mesocosm experiment suffered from interaction with pathogenic soil biota. Pathogen pressure affecting *L. terrestris* survival rate was also suggested in a paper based on a laboratory study, where mortality was observed to be unequally divided over batches and unrelated to treatment (Lowe & Butt, 2007).

We think that, with respect to *L. terrestris* colonisation, pathogen-pressure is an underexposed, but potentially very important, factor that deserves more scientific attention. After all, consideration of pathogen pressure may improve the various predictive models for *L. terrestris* occurrence and abundance that thus far show limited accuracy, for example models by Pelosi et al. (2008) and Lindahl et al. (2009). Pathogens may well play a role in the patchiness of earthworm abundance within a field or absence on field level; however, these questions are beyond the scope of this research.

4.6 Experimental set-up

4.6.1 Mesocosms

In earthworm research, mesocosms are a valuable tool for carrying out semi-controlled experiments. A mesocosm allows testing the set-up under 'normal' climatic conditions and in virtually undisturbed soil profiles, without running the risks of escape and predation. Mesocosms cover a substantial part of the gap between the laboratory and the field. However, the situation in a mesocosm does not exactly match field conditions; it remains a model. The physical presence of a mesocosm alters field conditions like predation, microclimate, air flow, temperature and freedom of movement and mate choice. Building upon our findings so far, the next step could be to move to field-scale and introduce *L. terrestris* into grassland where it is currently absent and monitor the development of the population for several years. Parallel to that, it would be interesting to explore the role of pathogens by running a controlled experiment using sterilised soil.

4.6.2 VIE tagging

VIE-tagging is a well-established method for marking animals that would be otherwise hard to identify. It is commonly used in research on amphibians and fish (Pine et al., n.d.; Hohn & Petrie-Hanson, 2013). In earthworm research, VIE-tagging has also been documented multiple times as an accessible and reliable method for

marking specimens without interfering with their potential for survival, maturation and reproduction (Gonzalez et al., 2006; Butt & Lowe, 2007) up to 27 months after inserting the tag (Butt et al., 2009). We did not notice any abnormalities about the earthworms after tagging them.

An inconvenience we discovered about VIE-tagging was that the VIE-tag inserted was often no longer visible from the outside. In most cases tags had migrated towards the posterior end of the body (Suppl. Figure S5.3A-C, S5.4A). Some of the tags had partly disintegrated (Suppl. Figure S5.3A-B, S5.4B). Consequently, all *L. terrestris* that were found at Location A needed to be dissected. Additionally, we had eleven earthworms with damaged tails, which brought about some uncertainty as it was impossible to be certain whether they had been tagged or not. To avoid overestimation, these are not included in the 15% retrieval. Within the group of tagged specimens recovered, 68% was NL and 65% was recovered from disturbed soil (Table 5.4).

5. Conclusions and future perspectives

This study aimed at gaining new insights into *L. terrestris* introduction into agricultural grasslands, mainly with respect to earthworm origin and suitability of the inoculated pasture. The results of the twelve-month mesocosm experiment at two organic farms show that it was possible for *L. terrestris* to survive and to produce offspring after inoculation. Uniquely, we introduced earthworms of two different origins (locally collected and commercially purchased from Canada) into two different grasslands (one where the species was already present and one where it was absent) with two different soil treatments (undisturbed versus hand-sorted soil with its residential earthworm population removed). The hypothesis that locally collected *L. terrestris* would outperform the Canadian specimens in survival, was only confirmed by the tagged earthworms at Location A. At Location B, the grassland without a resident *L. terrestris* population, there was no significant difference between the survival rates of NL and CA inoculum. In line with our hypothesis, more offspring was produced by NL than by CA inoculum, but only in undisturbed soil, there was no difference between offspring production of NL and CA in disturbed soil.

L. terrestris survival rate was not significantly affected by soil treatment (undisturbed versus loosened and hand-sorted where the resident earthworm population was removed), nor did we find any significant effects of soil treatment on offspring production. Refuting our expectations, negative interactions between the abundance of

residential species, *L. rubellus* in particular, and *L. terrestris* were absent. From this we conclude that introduced *L. terrestris* does not necessarily engage in interspecific competition when resources are abundantly available, allowing co-existence with the residential earthworm population.

Overall, we see potential for *L. terrestris* inoculations in agricultural grasslands where the species is absent or abundance is low. Such inoculation would take place preferably using locally collected or locally bred *L. terrestris*, as this reduces the environmental burden of transport and is likely to improve their chances of survival. Several parties are developing ways to breed *L. terrestris* in captivity. Although our results were obtained under semi-controlled conditions, they cannot simply be extrapolated to normal field conditions. The next steps would be to gain more insight into the effect of pathogens by running controlled experiments with sterilised soil as well as by executing long-term experiments in non-enclosed plots. These experiments will be needed to elucidate whether and how *L. terrestris* inoculation can be used as an ecological tool by farmers to improve water regulation of their grasslands.

6. Acknowledgements

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7. Author contributions

Roos van de Logt: conceptualisation, methodology, software, validation, formal analysis, investigation, data curation, writing – original draft, visualization. Prof. Dr. Nick van Eekeren: conceptualisation, methodology, validation, investigation, writing – review and editing, supervision, project administration, funding acquisition.

8. Funding

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9. Data availability

The datasets generated and analysed in this study are available from the corresponding author on reasonable request.

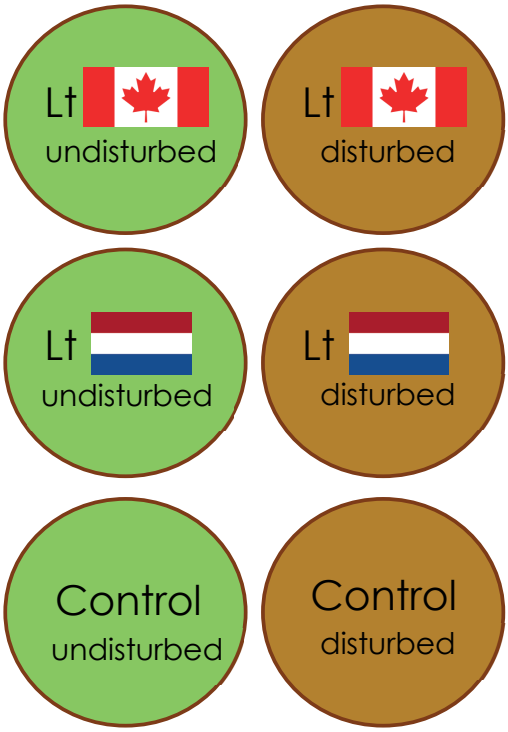
10. Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

11. Supplementary materials



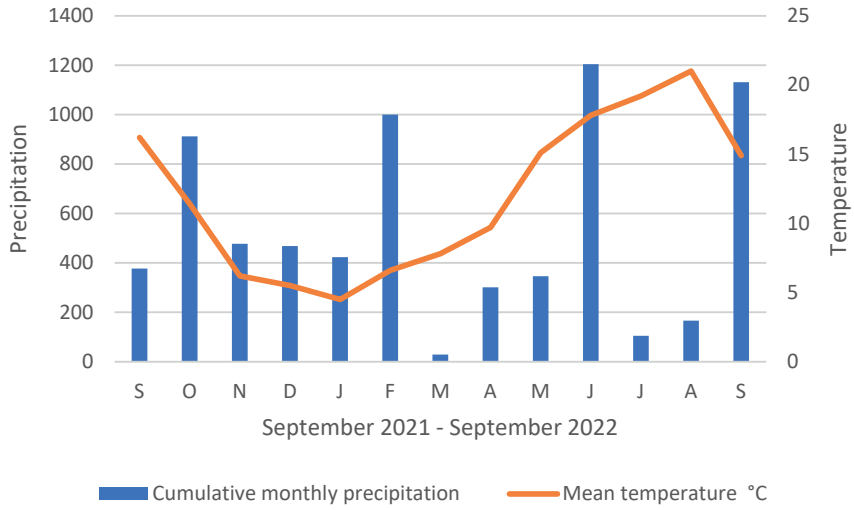
Supplementary Figure S5.1A Experimental set-up at Location A.



Supplementary Figure S5.1B Schematic overview of the six different treatments that were represented per block. Four blocks at location A, three blocks at location B. Lt = *Lumbricus terrestris*; undisturbed = soil was not manipulated; disturbed = resident earthworm population was removed through hand-sorting and mustard extraction, grass clover was resown (see materials and methods for more details on procedure); Canadian flag = inoculum was from Canadian origin; Dutch flag = inoculum was of local origin; control = no earthworms were introduced.

Supplementary Table S5.1 Manure analysis results

Quality	Result
Dry matter (g dm/kg)	222
Organic matter (g om/kg)	188
Nitrate (g N/kg)	6.03
C/N ratio	14
Ammoniacal nitrogen (g NH_3-N/kg)	1.5
Organic nitrogen (g N/kg)	4.5
Phosphorus (g P/kg)	0.83
Phosphate (g P_2O_5/kg)	1.90
Potassium (g K/kg)	8.2
Potassium oxide (g K_2O/kg)	9.9



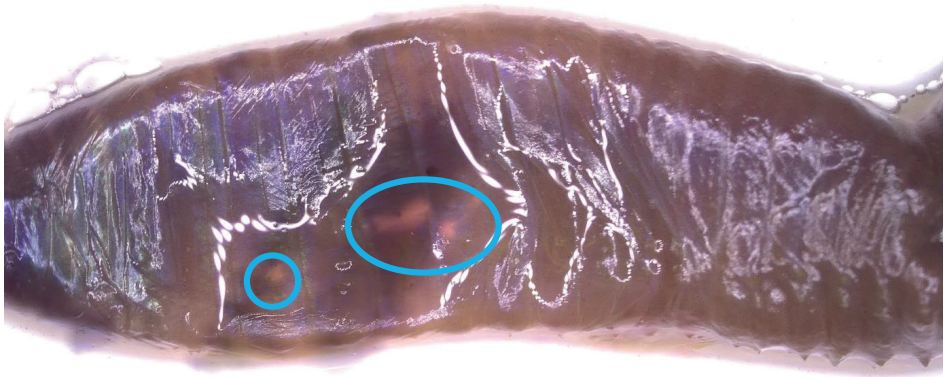
Supplementary Figure S5.2 Average temperature and cumulative rainfall as measured on a nearby (35 km) weather station.

Supplementary Table S5.2 Soil temperature (in °C) at 5, 10, 20, 50 and 100 cm depth at weather station De Bilt, located 60 km north of the experimental sites. The temperature was measured daily, four times per 24 hours (6:00, 12:00, 18:00 and 00:00); the temperature in the table is the average of all measurements in that month (https://www.knmi.nl/nederland-nu/klimatologie/bodemtemperaturen).

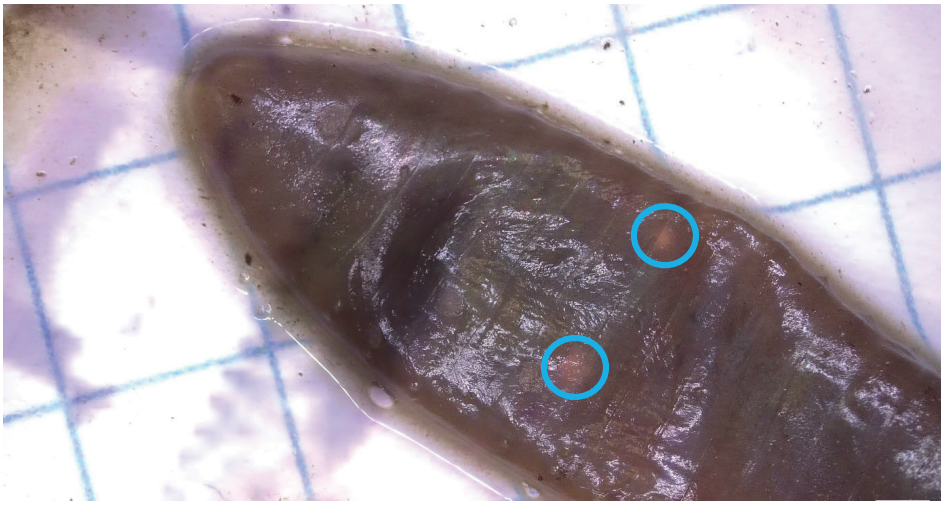
De Bilt	5 cm	10 cm	20 cm	50 cm	100 cm
Sep-21	17.0	17.1	17.0	17.0	16.6
Oct-21	13.1	13.2	13.4	14.0	14.5
Nov-21	9.6	9.8	10.1	11.1	12.0
Dec-21	6.7	6.8	7.1	8.0	9.1
Jan-22	6.4	6.4	6.7	7.4	8.2
Feb-22	6.6	6.7	6.8	7.3	7.8
Mar-22	6.6	6.7	6.8	7.1	7.5
Apr-22	9.0	9.0	8.9	8.8	8.7
May-22	13.8	13.7	13.2	12.4	11.4
Jun-22	17.1	16.9	16.4	15.4	14.1
Jul-22	18.7	18.5	18.0	17.1	16.0
Aug-22	19.5	19.5	19.0	18.1	17.1
Sep-22	16.3	16.5	16.5	16.8	16.7

Supplementary Table S5.3 General overview of species abundance, juvenile fraction and percentage of the total population. 'Unidentifiable' represents damaged individuals that could not be identified into species or age class. na = not applicable.

Species	Abundance (ind. m^{-2})		Juvenile fraction (%)		Percentage of total community (%)	
	A	B	A	B	A	B
L. castaneus	0.0	10	na	39	0	2.9
L. rubellus	22	124	97	62	4	36
A. rosea	7.1	0.0	80	na	1.3	0.0
A. chlorotica	53	4.6	34	50	9.6	1.3
A. caliginosa	260	172	63	46	47	50
A. limicola	24	0.0	89	na	4.4	0.0
L. terrestris	128	13	82	31	23	3.8
A. nocturna	4.0	0.0	68	na	0.7	0.0
Unidentifiable	52	22			9.5	6.4
Total	**550**	**345**	**68**	**51**	**100**	**100**



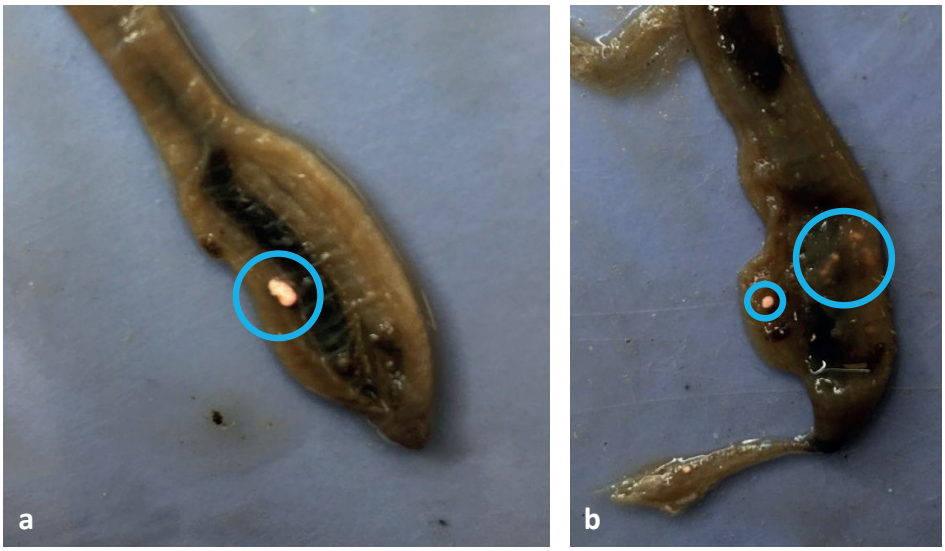
Supplementary Fig. S5.3A Picture of tagged and retrieved *L. terrestris* specimen. The tag appears to be partly disintegrated and migrated towards the posterior end of the earthworm.



Supplementary Fig. S5.3B Picture of tagged and retrieved *L. terrestris* specimen. The tag appears to be partly disintegrated and migrated towards the posterior end of the earthworm.



Supplementary Fig. S5.3C Picture of tagged and retrieved *L. terrestris* specimen.



Supplementary Fig. S5.4A and S5.4B Pictures of tags that were not visible through the skin of the earthworm, only after dissection.



Chapter 6

General discussion



1. Introduction

Earthworms make an important contribution to the water-regulating capacity of soils (Blouin et al., 2013; Deru et al., 2018). Water regulation has become an increasingly important ecosystem service, as climate change causes shifts in precipitation patterns, leading to an increased incidence of both peak rains and drought (Samaniego et al., 2018; Santos et al., 2016). Grassland is the most common type of land-use in the EU, with permanent grasslands covering 48 million hectares (30% of the total agricultural land area; 12% of the total land area) (Eurostat, 2018). Their sheer expanse means that grasslands play an important role in water regulation. In general, grasslands offer a stable and food-rich habitat for earthworms. In combination with a temperate climate – mainly sufficient moisture – and the absence of soil disturbance, this results in thriving earthworm populations that can reach densities of > 1,000 ind. m^{-2} (Hendrix et al., 1992; Rutgers et al., 2016). In turn, earthworm burrowing, feeding and casting behaviour support grassland quality through nutrient cycling, aeration, litter consumption and aggregate formation (Blouin et al., 2013; Van Groenigen et al., 2014). Especially deep-burrowing earthworm species, such as *Lumbricus terrestris*, contribute uniquely to **water regulation and climate adaptation**. In contrast to other earthworm species that occur in temperate grasslands, *L. terrestris* constructs burrows that are particularly deep and vertical (Jégou et al., 1999; Shipitalo & Gibbs, 2000). This type of burrow provides preferential flow routes for water infiltration and facilitates deeper plant rooting (Hallam & Hodson, 2020; Hoogerkamp et al., 1983; Willoughby & Kladivko, 2002). Water infiltration has great benefits for society, as it can replenish groundwater, which is vital to sustain ecosystem services provided by nature and agriculture, and it supports the prevention of surface runoff, which is not only beneficial to water quality but also averts soil erosion and flooding.

In the Netherlands, about 4,500 dairy farms and their grasslands are situated on sandy soil (estimation based on data from Jellema et al., 2025). Due to its relatively coarse texture, sandy soil is vulnerable to both drought and flooding. Therefore, it is imperative that water regulation in these soils is improved. The earthworm species *L. terrestris* is known to be able to play a role in this. However, its current distribution and the factors that drive the presence and abundance of *L. terrestris* are not well understood, the effects of different grassland renewal strategies on earthworm population dynamics in grassland remain understudied, and targeted inoculation of soils without an indigenous *L. terrestris* population have received too little scientific attention to offer a practical perspective. To address these matters,

three research objectives were set in this thesis, all within the context of grassland on sandy soil:

1. Elucidate how soil texture, groundwater level, *Lumbricus rubellus* population density and grassland age affect the presence and abundance of *L. terrestris*. This was mainly covered in **Chapter 2**, but was also touched upon in **Chapters 4 and 5**.
2. Learn more about the effects of different grassland renewal strategies on the resident earthworm population. This objective was covered in **Chapter 3**.
3. Explore if and how *L. terrestris* can establish a viable population after being inoculated into a grassland. This was covered in **Chapters 4 and 5**.

2. Main findings and advancing insights

This section starts with a summary of the main findings of each chapter. As these findings have been discussed extensively in Chapters 2-5, I dedicate the remainder of this section to a number of advancing insights that are relevant for the research objective of this thesis and that have emerged from the combined observations of the separate studies. I also present some new data which found no place in previous chapters, but which adds to our understanding of earthworm population dynamics in relation to grassland management in the context of my research objectives. **Opportunities and implications for future research** are included in the text.

2.1 Main findings – A summary

Chapter 2 describes a correlative study for which a field inventory was conducted among grasslands on sandy soil, aimed to explore the presence and abundance of *L. terrestris* in relation to soil texture, hydrology, interspecific competition with *L. rubellus* and grassland age. *L. terrestris* was found to be present under various soil textures, groundwater levels, species assemblies and grassland ages. Its abundance showed a positive correlation with soil loaminess at 30-40 cm depth and a negative correlation with the abundance of *L. rubellus*. *L. terrestris* showed a slight preference for soils with a lower maximum groundwater level. No significant effect of grassland age was found.

Chapter 3 presents an on-farm mesocosm experiment, repeated in two consecutive years. We tested the effect of several grassland renewal strategies on the resident

earthworm population, namely strategies with or without tillage and with or without vegetation cover loss. Six months after application we considered the effects of these strategies on abundance, demographic composition and species diversity. We found a clear effect on earthworm abundance, which decreased along a gradient of increasing disturbance. Tillage had a stronger negative effect on earthworm abundance than soil cover loss, and their combined effect was most detrimental. Especially endogeic species (*Aporrectodea caliginosa* and *Aporrectodea rosea*) strongly decreased in abundance when tillage was applied and when soil cover was lost; the effect was smaller among epigeics and anecics. The number of adult earthworms six months after treatment application was more strongly affected by tillage and soil cover loss than the number of juveniles. Species diversity did not vary between treatments.

In **Chapters 4 and 5**, we again zoomed in on *L. terrestris*. **Chapter 4** describes an on-farm mesocosm study. *L. terrestris* was inoculated into grassland at two different farms that did not have a resident *L. terrestris* population, but that seemed suitable for one, based on insights from literature and the inventory in Chapter 2. It was found that *L. terrestris* was able to survive, burrow to a depth of > 60 cm and produce offspring during a period of 15 months. Survival rate was 32% after seven months and 6% after 15 months. There were indications of interspecific competition between *L. terrestris* and *L. rubellus* at the expense of *L. terrestris*. We proposed several possible reasons that could explain why *L. terrestris* survival rate was low. These were drought, as there was a dry summer, and competition for food with *L. rubellus*. In addition, we suspected a low adaptability of the commercially purchased inoculum to its new environment. **Chapter 5** describes a follow-up inoculation experiment with a similar mesocosm setup. Here, we compared *L. terrestris* inoculum from two origins, local and imported from Canada. *L. terrestris* was introduced into two grasslands; one already had a resident *L. terrestris* population, the other did not. The grassland with a resident population was selected to enable us to separate the effects of inoculation from suitability of the habitat. As in Chapter 4, *L. terrestris* survived, burrowed to > 60 cm deep and produced offspring in a period of 12 months. The survival rate was highest at the farm without a resident population; 26% versus 15%. On average, the local inoculum performed slightly better than the Canadian inoculum.

2.2 Advancing insights: Are sandy soils a bridge too far for *L. terrestris*?

Much of this thesis centres around grassland on sandy soil in the provinces of Overijssel, Gelderland and Noord-Brabant in the South-East of the Netherlands. We specifically

chose this area and its associated sandy soil because it is prone to both drought and flooding, which is becoming increasingly problematic in the face of shifting precipitation patterns. In principle, earthworms, and especially *L. terrestris*, can help to improve the water-regulating capacity of these soils. However, a paradox arises; on the one hand, *L. terrestris* can improve water regulation and drought resistance of sandy soil, but on the other hand, the species depends on sufficient soil moisture for survival. This creates a field of tension, because the soils that might benefit most from *L. terrestris* activity may also be the soils that are less suitable for it. In this section I explore the potential of grassland on sandy soil as a habitat for *L. terrestris* populations in relation to the results from Chapters 2, 4 and 5 as well as other studies.

A national inventory of grasslands at Dutch dairy farms (n = 358) showed that anecic earthworms were present in 22% of 192 sampled farms on sandy soil. For marine clay, this was 32%, for river clay 29%, for peat 7% and for loess 27% (Van Eekeren et al., 2014). Two anecic species were found, *Aporrectodea longa* and *L. terrestris*, of which the latter is far more common. Although anecics were found to occur most often on marine clay, curiously, the highest *L. terrestris* density of the whole inventory (317 ind. m^{-2}) was found on a sandy soil (BoBi, unpublished). These results sparked interest in further exploration of the potential role of *L. terrestris* in grasslands on sandy soil, with the idea of inoculation as a logical further step.

Our inventory demonstrated the presence of *L. terrestris* at 60% of the sampled locations (Chapter 2). Sixty percent seems high compared to the national inventory (22% on sandy soil), which may be due to a difference in sampling method. In the national inventory, only hand-sorting of soil cores was applied, whereas we complemented hand-sorting with mustard extraction, which is a more effective way to sample anecic earthworms (Pelosi et al., 2009). Therefore, the national inventory may have yielded some false negatives for *L. terrestris* presence. Alternatively, it is possible that we found a larger presence of *L. terrestris* because the sandy soils that were sampled in Chapter 2 were relatively rich in loam (min 7%; max 40%; on average 24%). In previous studies, *L. terrestris* has been associated with finer textured soils (Lindahl et al., 2009). Sandy soil is more prone to drought than loam or clay soils, which have a better water- holding capacity (MAAF, 2002). It is mostly for this reason that sandy soil is considered a less suitable habitat for *L. terrestris*, and for earthworms in general (Curry, 2004; Hendrix et al., 1992). The role of soil moisture in relation to texture and its consequences for *L. terrestris* survival is discussed in more detail in section 3.2.

Except for the indirect impact of the coarseness of sandy soil on *L. terrestris* via soil moisture, its texture can also affect *L. terrestris* directly. In a laboratory experiment by Hawkins et al. (2008), it was found that *L. terrestris* was able to cross a 3 cm sand layer and establish a burrow. However, it took these earthworms 1.7-5 times longer to establish their burrows than controls on loam. Survival was lower among earthworms that had to cross sand (89% compared to 100%). Some earthworms were torpid, and the authors hypothesise that these earthworms may have been weakened from the effort of burrowing in sand. Additionally, it was observed that the earthworms that died had bloody mouths; they had possibly been injured by the abrasive sand. One dead earthworm was found with a large sand particle lodged in its mouth. These findings seem to suggest that ingesting – or attempting to ingest – large or abrasive soil particles can be harmful and even fatal to *L. terrestris*. *L. terrestris* may not always be able to assess the 'ingestability' of a soil particle before attempting to do so. This could lead to injured mouthparts, or even congestion, as seemed to have happened in the experiment by Hawkins et al. (2008), which could compromise *L. terrestris* abundance.

Projecting this hypothesis onto our own findings in Chapters 4 and 5, part of the inoculum may have died because they ingested large particles that injured them or were fatal. The originally *L. terrestris*-free soils in the in-field mesocosm experiments contained around 85% sand (exact percentages per location specified in Chapters 4 and 5), which may have held particles that were too large and/or abrasive for *L. terrestris*. Unfortunately, it is unknown when and how the inoculated earthworms died, as is elaborated upon in section 3.1. In future research, a small and controlled mesocosm setup with soil from the field could allow for close monitoring of *L. terrestris* survival as well as inspection of the mouthparts if earthworms die. Interestingly, the suitability of a sandy soil for *L. terrestris* may thus also depend on the coarseness or the amount of large and abrasive particles that are present within the sand fraction.

In summary, field inventories clearly demonstrate that sand is not necessarily a limiting factor as such. However, I think it is unlikely that all sandy soils are suitable for hosting an *L. terrestris* population. In the category of sandy soils, it may be coarseness and particle size that facilitate or hinder *L. terrestris* populations, but further research is required to verify this. Hence, I conclude that sandy soil is not necessarily 'a bridge too far' for *L. terrestris*. Existing populations in sandy soils should be managed well and research into inoculation of *L. terrestris* in seemingly suitable grasslands merits further exploration.

2.3 Advancing insights: Suitability versus accessibility of studied grasslands

The inventory showed that the presence and abundance of *L. terrestris* was heterogenous and difficult to pinpoint, even within the narrow context of temperate grasslands on sandy soil located no more than 15 km apart and with virtually no variation in elevation. The agricultural landscape in our research area was characterised by plots of 0.5 to 3 ha in size. Grassland dominated over arable land, and in various places it was interspersed with landscape elements such as trees and small forest patches. Ditches were present, but not between each plot, and most were not wider than 1 m. A small river, the Zandleij, ran through the area. Quite a few tarmac roads veined the region, but most were no wider than 5 m (appendix, Figure x). Most grasslands were used intensively (receiving 250-320 kg N ha^{-1} annually and being either grazed or mown 4-7 times per year), but there were also some extensively used and natural grasslands. We hypothesised that this landscape would be favourable to *L. terrestris*. However, this was not fully confirmed by the data: although *L. terrestris* was found in 60% of the sampled grasslands, it was only abundant (> 50 ind. m^{-2}) in 31% of all grasslands (Chapter 2).

The inoculation experiments showed that *L. terrestris* can survive and produce offspring in grasslands that had no population of the species prior to inoculation. However, the experiments also showed that 74-94% of the inoculum died within 15 months. These outcomes from Chapters 2, 4 and 5 are paradoxical; on one hand they show that *L. terrestris* does not occur everywhere (Chapter 2) and is not able to survive everywhere (Chapters 4 and 5), yet on the other hand, they show that *L. terrestris* populations can thrive in a variety of temperate grasslands on sandy soil (Chapter 2) and survive, burrow and produce offspring within 12 months after inoculation into grassland on sandy soil that had no indigenous *L. terrestris* population (Chapters 4 and 5). This raises the question *why would L. terrestris not occur in grasslands that are seemingly suitable*? Within the context of grasslands on sandy soil in the Netherlands, I hypothesise that there are two possible answers to this question: (1) the grassland is in fact unsuitable for *L. terrestris* settlement and reproduction, and (2) the habitat is suitable, but not easily accessible to *L. terrestris* and thus not, or not yet, colonised. In the latter case, 'colonisation' may also be 'recolonisation' after the disappearance of the original *L. terrestris* population, which may have happened during extreme drought events or the continuous use for arable cropping. Below I discuss both options with respect to each of the experiments.

If it is indeed **suitability** that determines whether *L. terrestris* is present or absent in a grassland, I would assume that *L. terrestris* occurred in all suitable grasslands in the

research area. As none of the factors we measured in Chapter 2, nor a combination of them, was enough to explain the presence and abundance of *L. terrestris*, they must be explained by factors that we did not measure. The factors measured in Chapter 2 were soil texture, groundwater level, *L. rubellus* abundance and the number of years since last mechanical soil disturbance; please note that seasonal variety in abundance was overcome by sampling in early spring, when earthworms are generally relatively abundant. Certain abiotic soil factors that we did not discuss, which in some studies have been found to strongly correlate to *L. terrestris* abundance in temperate forest, are K^+, Mg^{2+}, Ca^{2+}, pH, CEC (Ransom & Billak, 2015), SOM and exchangeable Ca (Yatso & Lilleskov, 2016). SOM and pH were also analysed at 0-10 cm and 30-40 cm depth for the soil samples that we took in Chapter 2, but no correlation with *L. terrestris* abundance was found ($r^2 < 0.025$ for each of the four correlations). It is likely that biotic interactions with microorganisms, either beneficial or harmful, also shape *L. terrestris* communities. Hypothetically, the absence of *L. terrestris* could be explained by the presence of a certain pathogen, or the absence of microorganisms that are vital for *L. terrestris* colonisation, for example because they support digestion or reproduction. Future research should investigate the relationship between earthworms and beneficial or harmful microorganisms, as there is currently very little knowledge about this potentially impactful interaction. Overall, we are not the first to be unable to pinpoint where *L. terrestris* is absent or present, as more studies report that *L. terrestris* dispersal is restricted by unknown factors (Holdsworth et al., 2007; Stoscheck et al., 2012).

With respect to the inoculation experiments, it is not possible to draw a long-term conclusion about the suitability of the grasslands, because the experiments ran for only 15 and 12 months (Chapters 4 and 5, respectively). Still, I can positively state that the mesocosm setup within these grasslands was suitable enough to facilitate the survival, burrowing and offspring production of 6-26% of the inoculated *L. terrestris* specimens during the running time of the experiment. This means that there were no acute fatal conditions and that these grasslands were able to provide at least the basic requirements for 12-15 months of survival, burrowing and offspring production.

If it is not suitability but **accessibility** that explains why *L. terrestris* was absent from 40% of the sampled grasslands of the inventory (Chapter 2), this could be due to limitations in dispersal or in time. The former can be divided into natural and anthropogenic dispersal. Both have been discussed in detail in Chapter 1. The latter, time, could be an important factor when the presence of *L. terrestris* in the area is discon-

tinuous. For example, Lindahl et al. (2009) state that land-use is the most important factor in determining the presence and abundance of *L. terrestris*, which has a strong preference for perennial land-use such as is common in grassland. However, in the past decades, land-use has changed. For this reason, some of the grasslands that we sampled may have had a history of intense tillage, pesticide application and arable cropping, and these could all have contributed to *L. terrestris* population collapse at the field scale (Astaykina et al., 2022; Stroud et al., 2016). Given the generally slow natural dispersal and physical barriers such as waterways and roads in the landscape, recolonisation of these fields might take many years. Anthropogenic dispersal could speed up the process; however, considering the limited survival rates of the inoculation experiments (6-26%, Chapters 4 and 5) combined with a slow reproduction cycle, it may well take many years before anthropogenic-mediated recolonisation reaches a level where *L. terrestris* is present in the whole grassland and its population has a substantial density. This time path is not the preferred option for farmers and water boards that would like to benefit from the improved water regulation that *L. terrestris* activity can bring.

In summary, I suspect that the absence of *L. terrestris* from grasslands on sandy soil is not necessarily an indication that the grassland is unsuitable for hosting the species. The absence of *L. terrestris* could also be explained by slow *L. terrestris* dispersal over time. As a consequence, if effective inoculation strategies can be developed with a survival rate that is higher than that which was found in the mesocosm experiments of Chapter 4 and 5, targeted inoculation could help to establish *L. terrestris* populations in grasslands that are out of reach for existing and expanding populations. Adequate land management can support and accelerate the growth of new and existing *L. terrestris* populations. Speeding up the natural process is desirable, given the urgency of climate adaptation.

2.4 Advancing insights: Recovery of earthworm populations after disturbance

The recovery of earthworm populations after a disturbance was a recurring topic throughout the chapters, most explicitly in Chapter 3, where we tested population dynamics in response to various combinations of tillage and soil cover. In Chapter 2, recovery after a disturbance was included as grassland age (defined by the number of years since the last mechanical disturbance). In Chapters 4 and 5, half of the mesocosms for the inoculation experiments were disturbed when the soil was hand-sorted to a depth of 60 cm and resident earthworm populations were removed

(referred to as 'hand-sorting' in short). Recovery of the resident population was not a research question in Chapters 2, 4 and 5, and as such it was not discussed. Nevertheless, we collected some interesting and surprising data regarding population recovery, which will be presented here. First, I evaluate the value of 'grassland age' as a predictor for earthworm abundance (Chapter 2), then I go into population recovery data from the hand-sorted mesocosms (Chapters 4 and 5), and finally, I discuss how these findings shine a new light on the interpretation of our conclusions in Chapter 3.

The **correlation between total earthworm abundance and grassland age** in Chapter 2 was very weak ($r^2 = 0.066$). Although this did not confirm our hypothesis that a longer period without mechanical soil disturbance would promote earthworm abundance, the weak correlation that we found is not unusual. Earthworm abundance recovery is a function of the interplay between various factors, such as land-use history, land management and weather conditions. This is also reflected in ambiguous relationships between grassland age and earthworm abundance as reported in other studies. Fox et al. (2017) found a positive correlation between earthworm abundance and number of years since last tillage event in a clay loam soil. Wodika et al. (2014) found a linear increase in earthworm abundance with time in a tallgrass prairie. However, also in a tallgrass prairie, Hensley et al. (2021) described a decline in earthworm abundance over a period of 12 years. Van Eekeren et al. (2008) reported greater earthworm abundance in 36-year-old grassland than in three-year-old grassland. Hoeffner, Santonja et al. (2021) found that soil properties explained more of the variation in the earthworm community than grassland age. In line with this, we found that the percentage of loam positively correlated with total earthworm abundance ($r^2 = 0.498$). Thus, the effect of grassland age is strongly contextual and in itself not a good predictor of earthworm abundance.

Figures 6.1A-E show the level of **population recovery after soil disturbance and earthworm removal by hand-sorting** after 8 months (Chapter 4, Figure 6.1A and 6.1B) and 12 months (Chapter 5, Figure 6.1C, 6.1D and 6.1E). At both locations of Chapter 4, earthworm abundance after 8 months approached the level of the mesocosms without the intervention. This applied to all ecological groups and is similar for adults and juveniles (Figure 6.1A and 6.1B). In Chapter 5, in a comparison of the two locations, the earthworm abundance and biomass had halved at location A, whereas full recovery had occurred at location B (Figure 6.1C, 6.1D and 6.1E).

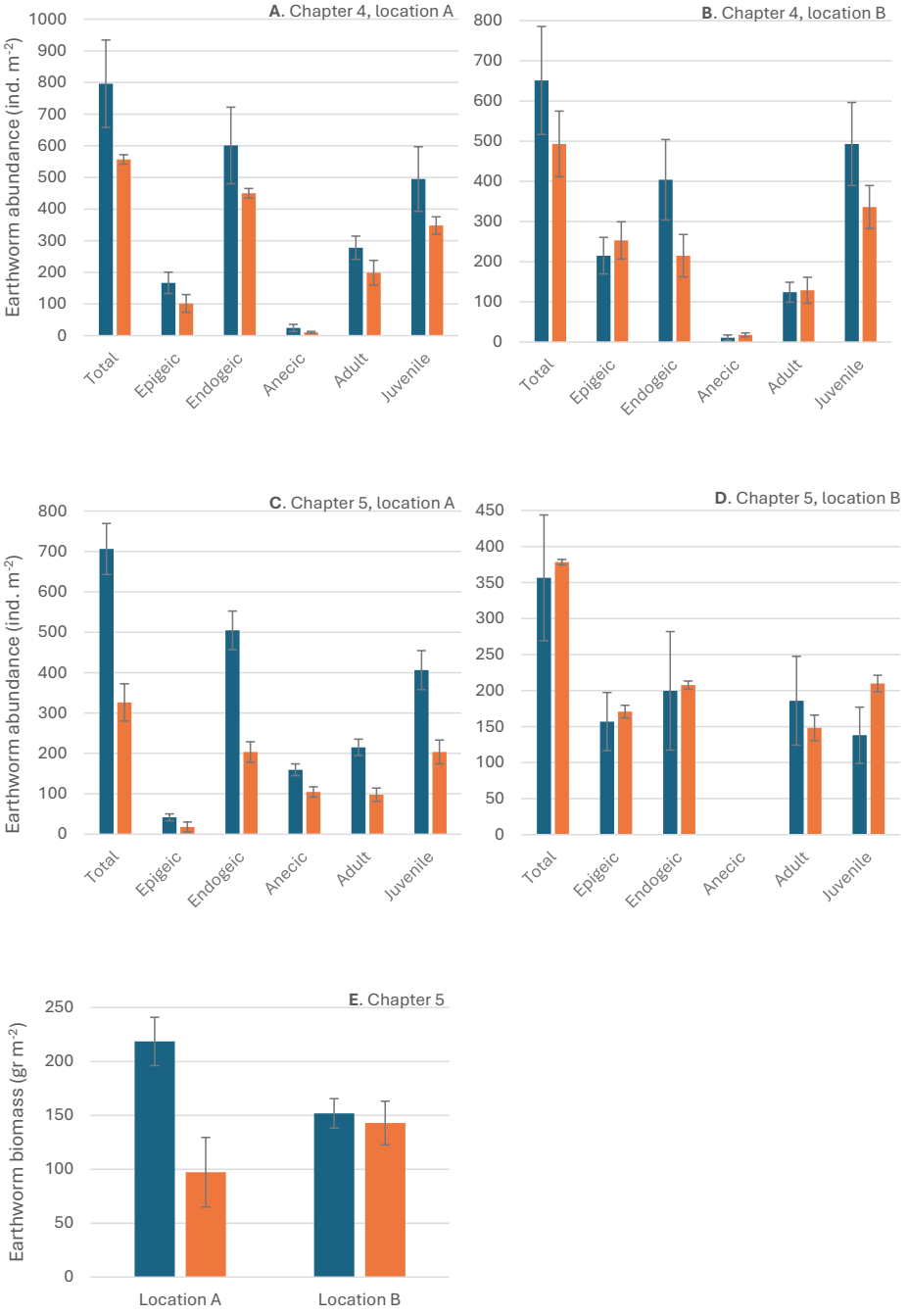


Figure 6.1 Earthworm abundances and biomass in undisturbed (blue) and hand-sorted (orange) mesocosms in the inoculation experiments of Chapters 4 and 5; please note that locations A and B from Chapter 4 are not the same locations as A and B from Chapter 5. Graphs are based on control mesocosms, and these were not inoculated with *L. terrestris*. Disturbed and undisturbed mesocosms that are paired were always harvested at the same moment. Biomass data per location per treatment was only available for Chapter 5.

It was surprising to find that earthworm populations in hand-sorted mesocosms had almost fully recovered at three of the four experimental sites. The hand-sorted mesocosms were virtually devoid of earthworms at the onset of the experiment: only cocoons were left behind, and possibly some juveniles had accidentally been overlooked. We had not expected that the population would be able to recover from such a severe disturbance within 8-12 months. Below, I mention three factors that could have facilitated this quick recovery and then zoom in on endogeics, epigeics and anecics separately.

1. **Timing** may have been a factor, as hand-sorting took place in spring (April), a time of the year when there are usually quite a number of juveniles and cocoons present in the soil. Presumably, the few earthworms that were left behind and the juveniles that later hatched from the cocoons benefited from the post-hand-sorting conditions in the mesocosms.
2. The mesocosms offered a **resource-rich environment**. Food was ample because manure and leaf litter were added to all mesocosms; in addition, the sward was torn into small pieces during hand-sorting and thereafter returned into the mesocosm together with the rest of the 0-20 cm layer, thus resulting in extra input of organic matter in the 0-20 cm layer.
3. The nets that covered the mesocosms **protected the earthworms from predators**.

Taken together, these factors may have created favourable conditions for quick population growth. Below, I discuss population recovery among endogeics, epigeics and anecics separately.

When comparing Figure 6.1A and 6.1B, it seems that the **endogeic** population at location A recovered better in both a relative and absolute sense than at location B. At location A, the endogeic community was dominated by *Allolobophora chlorotica*, whereas it was dominated by *Aporrectodea caliginosa* at location B. Under the prevailing conditions, it is possible that *A. chlorotica* could deal better with the disturbed soil and incorporated sward than *A. caliginosa*. At location A of Chapter 5, the endogeic population was also dominated by *A. caliginosa*, which also recovered poorly from the hand-sorting (Figure 6.1C). *A. caliginosa* was found to respond negatively to soil disturbance in Chapter 3 as well, as was also evident in various other studies (Marinissen, 1992; Pérès et al., 2010). However, these observations are challenged by our findings at location B in Chapter 5 (Figure 6.1D). Again, *A. caliginosa*

was the dominant endogeic species, but here it recovered fully from hand-sorting (Figure 6.1D). Local conditions might explain why *A. caliginosa* recovered better at location B than at location A. Alternatively, recovery may also be approached from a different perspective: not relative to the original abundance but in absolute numbers. After all, we do not know if the number of cocoons and overlooked earthworms was relative to the resident population or similar in absolute numbers across locations. In the latter case, *A. caliginosa* recovered equally well at location A and B in Chapter 5, to an abundance of ± 200 ind. m^{-2}. If earthworm population recovery after disturbance is better described in absolute than in relative terms, it would have considerable implications for the level of population recovery that can be expected after disturbance.

Epigeic earthworms were scarce at location A in Chapter 5, but recovered well at location B and at both locations in Chapter 4. All mesocosms received manure and leaf litter, both of which are good food sources for epigeics, thus providing the energy required to grow and reproduce. At all four experimental sites, epigeics were almost exclusively represented by *L. rubellus*, a species known to have a relatively quick metabolism and reproduction cycle (Butt, 1998; Uvarov, 2009). These characteristics, combined with the input of manure and leaf litter as food resources, could explain the quick recovery of the population. **Anecic earthworms** were only present in considerable abundance at location A in Chapter 5 and recovery after hand-sorting was high, with a mean of 152 ind. m^{-2} in undisturbed controls versus a mean of 105 ind. m^{-2} in disturbed controls. The input of food sources in the form of litter and manure may have contributed to population recovery; a more detailed discussion can be found in Chapter 5.

Interestingly, the results on population recovery in Chapters 4 and 5 offer an additional perspective on our conclusions in Chapter 3. Population recovery was generally high in Chapters 4 and 5, and, as discussed above, this seemed to be facilitated mainly by the ample food availability above- and below-ground, combined with a protective environment. The soil disturbance that was inherent to hand-sorting apparently did not hinder recovery. In Chapter 3, recovery was generally 50% or less for the GrassTill and BareTill treatments, which was poor compared to GrassNoTill (Figure 6.2A and 6.2B). The populations in Chapters 4 and 5 had more time to recover (8 and 12 months versus 6), but still, the findings seem to indicate that earthworm populations recovered better from active removal of the population than from tillage. This was surprising, as the former appears far more rigorous than the latter. The protec-

tive environment of the mesocosms was identical across all three experiments. This may indicate that food availability could explain the difference in recovery level. In Chapter 3, the grass sod was removed in all treatments except GrassNoTill and no extra resources were supplied, whereas these were supplied in Chapters 4 and 5 (manure, litter and return of the deconstructed sod). This seems to indicate that when food is abundant, earthworm populations in grassland can recover well from severe population decline and soil disturbance. However, we concluded in Chapter 3 that soil disturbance through tillage was more damaging to earthworm abundance than loss of vegetation cover. This was true under the given circumstances, but the findings from Chapters 4 and 5 suggest that recovery rates may well have been higher if the grass sod had not been removed. The practical implications for grassland management will be discussed in section 4.1.

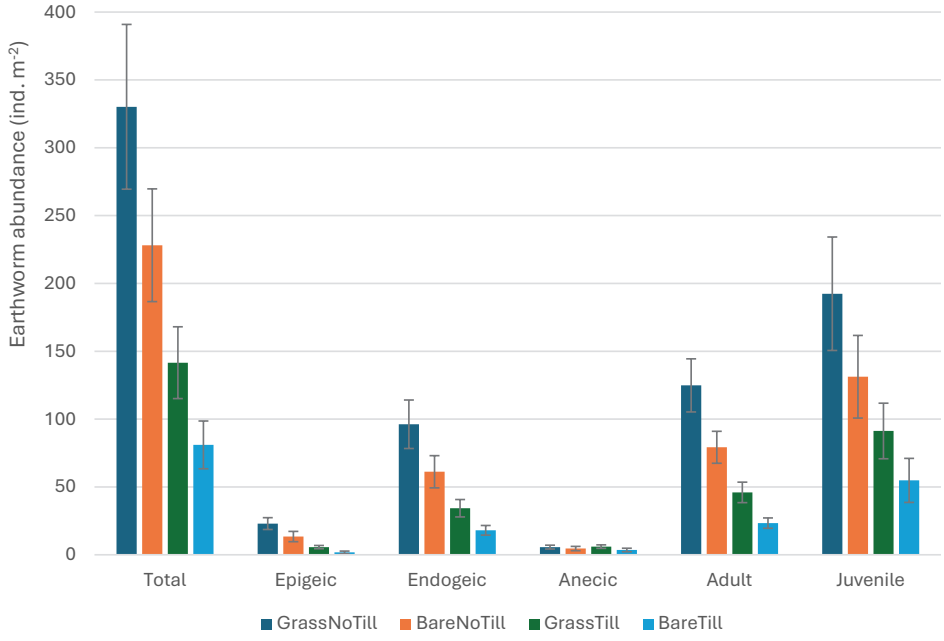


Figure 6.2A Mean earthworm abundance per treatment across both years of the mesocosm experiment in Chapter 3. GrassNoTill = continued grassland; BareNoTill = grass sod was removed and not reseeded, no tillage; GrassTill = grass sod was removed, tillage was applied, then grass was reseeded; BareTill = grass sod was removed and not reseeded, tillage was applied.

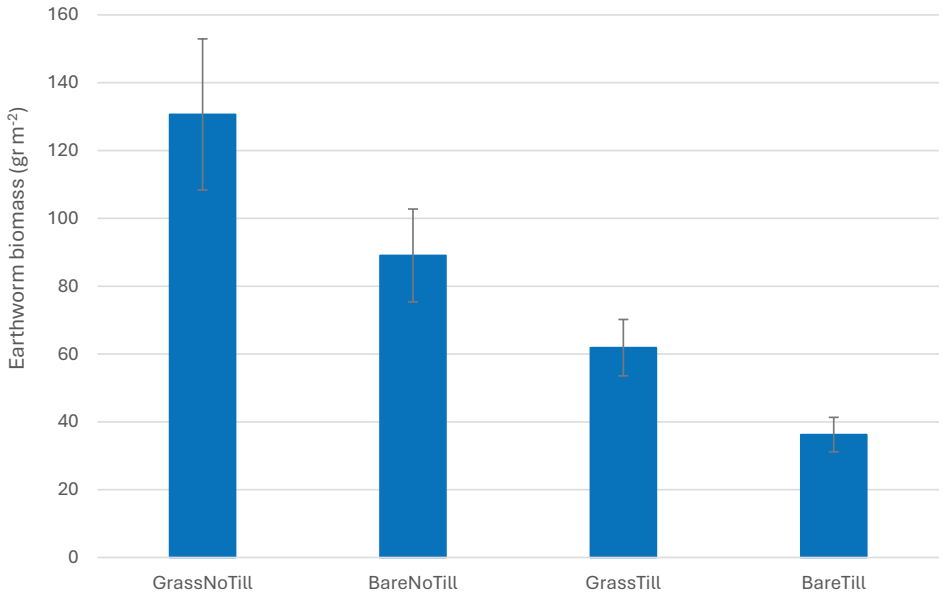


Figure 6.2B Mean earthworm biomass per treatment across both years of the mesocosm experiment in Chapter 3. GrassNoTill = continued grassland; BareNoTill = grass sod was removed and not reseeded, no tillage; GrassTill = grass sod was removed, tillage was applied, then grass was reseeded; BareTill = grass sod was removed and not reseeded, tillage was applied.

In conclusion, earthworm population recovery after disturbance in grassland on sandy soil depends on various factors, such as weather conditions, food availability, food distribution, predation, and timing of the disturbance. Not all species recover equally or equally fast. The absence of food has a negative effect on population recovery. Under beneficial circumstances, some populations are able recover quickly from severe disturbance.

3. Methodological challenges and opportunities

In this section, I review the strengths and weaknesses of the experimental approaches that were used in this thesis. I discuss how they may have affected the outcomes of the experiments and what the consequences are for the interpretation of our findings. Some recommendations for alternative or complementary experimental approaches future research are provided.

3.1 General difficulties in studying earthworms

A recurring difficulty when studying soil organisms is the unavoidable fact that they live in the soil. As a consequence, the study subject resides in a non-transparent matrix and cannot be studied in direct interaction with its living environment without being disrupted. This makes it very challenging to observe the natural behaviour of soil organisms such as earthworms. Consequently, it was impossible to closely monitor the inoculum during the experiment (Chapters 4 and 5). We know how many *L. terrestris* were introduced and how many had survived until the moments of harvest. Unfortunately, these snapshots provide no information about why and when the rest of the inoculum died. Therefore, it remains challenging to identify the factors that affect *L. terrestris* survival and reproduction, and the same applies to predicting the future development of the inoculated population.

In all four experiments (Chapters 2, 3, 4 and 5), we used a recommended and widely applied but destructive earthworm sampling method: hand-sorting of a soil core, combined with mustard extraction from the soil pit (Pelosi et al., 2021; Singh et al., 2016). It was not possible to take soil cores from the mesocosms to **monitor survival throughout the experiment**. Practical restrictions limited the maximum size of the in-field mesocosms; they were ø 61 cm (Chapters 3, 4 and 5) and taking a soil core would cause too much damage. Instead, all soil from each mesocosm underwent hand-sorting and mustard extraction in one go, at the harvest of the experiment. In attempting to monitor *L. terrestris* establishment after inoculation, I tried midden-counting as a proxy for *L. terrestris* presence. Middens had been constructed in the mesocosms, indicating at least some *L. terrestris* activity. However, it proved difficult to count middens under the growing grass and it was difficult to see where one midden ended and the next began. Also, surface activity of *L. terrestris* is seasonal and variable (Butt & Nuutinen, 2024; Grigoropoulou & Butt, 2012), and middens can persist without being occupied (Aira et al., 2009). The number of middens is thus not necessarily representative of the number of individuals present (Grigoropoulou & Butt, 2012). I suggest that future research moves towards non-destructive methods for monitoring survival of the inoculum.

Ecoacoustics is an example of an emerging, non-destructive method for monitoring earthworm population dynamics (Lacoste et al., 2018; Stroud et al., 2025). The method involves using sound and vibrations to monitor belowground biological activity and soil health. Another option could be analysing **environmental DNA** (eDNA). The combination of eDNA and metabarcoding is currently used in soil

biodiversity research (Sapkota et al., 2025). Still, at present it is highly doubted whether eDNA methods will ever be able to generate quantitative data. A low-tech and low-cost method for non-destructive in-field earthworm monitoring could be to temporarily place a piece of **wetted cardboard** or wetted carpet on the soil (a suggestion based on personal communication with an earthworm-breeder). *L. terrestris* needs to surface in order to collect food and find a mate. The time that it can safely spend on the surface is limited by drought, risk of predation (Onrust & Piersma, 2017) and sunlight, as UV light is harmful to earthworms (Chuang et al., 2006). However, under wetted cardboard or carpet, the earthworms are protected from all three. Under safe conditions, *L. terrestris* can spend more time on the soil surface, and it also does so during daylight hours, which makes it possible to count the earthworms at regular intervals over the course of the experiment. A possible disadvantage of this method is that it gives the earthworms more chance to mate and feed, which may artificially increase their survival and reproduction rate compared to normal conditions in the field. To mitigate this effect, the cover should only be used periodically. Still, this may be a simple but effective way to monitor *L. terrestris* survival rate after inoculation and to monitor populations in general.

3.2 Soil moisture measurements

In each of the experiments that we executed, **proxies** such as soil texture (Chapter 2) and precipitation data from the nearest weather station (Chapters 3, 4 and 5) were used as an estimator for soil moisture. An important advantage of soil texture and precipitation data is that they are easy to obtain and that they provide information about a long period of time. Under normal circumstances, soil texture does not change over decades and precipitation data from weather stations in the Netherlands since 1901 is reliable and freely accessible. By contrast, a soil moisture measurement only reflects a particular point in time. However, I think there would be added value in measuring the actual soil moisture and soil temperature at the experimental site. Moreover, it could be interesting to measure the soil's water holding capacity, as this gives a better indication of a soil's proneness to drought. Ideally, in future experiments, moisture sensors could be installed within mesocosms to keep track of moisture during the course of the experiment. In a sampling campaign such as the one that was executed for the inventory (Chapter 2), soil moisture and water holding capacity could then be determined for each sample.

Although precipitation and soil texture are both important factors determining soil moisture, they remain proxies, thus compromising the extent to which the effect of soil moisture on the collected earthworm data could be determined. In Chapters 4 and 5, I discussed the fact that drought – due to the dry summers in both experiments – could be a reason for the high mortality rate of the inoculum (74-94%). Taking loaminess as a proxy for soil moisture retention (MAAF, 2002), it seemed plausible that drought would have been a limiting factor for *L. terrestris* survival because in both experiments, the location with the highest percentage of loam had the highest abundance of *L. terrestris*. As mentioned above, soil moisture as such was not monitored on a regular basis, but a gravimetric soil moisture measurement was performed in the spring of 2021, during the second inoculation experiment (Chapter 5) (Table 6.1). It reflected what we had expected: the loamier soil had a higher soil moisture content. This supports the hypothesised positive relationship between soil texture, soil moisture and *L. terrestris* abundance in sandy soil. Closer monitoring, as suggested above, could give a more precise indication of the relationship between soil texture, soil moisture regime and earthworm abundance.

Table 6.1 Gravimetric measurement of soil moisture content. Samples were taken on the same day at both locations (May 6th, 2021, during the inoculation experiment described in Chapter 5)

	Soil moisture (%)	
Depth (cm)	**Location A (35% loam)**	**Location B (19% loam)**
0-10	15.1	15.7
10-20	13.5	10.3
20-30	15.2	9.8
30-40	14.3	10.5

Surprisingly, in the inventory (Chapter 2), we found an abundant *L. terrestris* population on very sandy soil (at 0-10 cm depth: 1% lutum; 11% loam; 85% sand; 4.4% OM). This was unexpected, because sandy soils with low loam content are usually more prone to drought (Yu et al., 2023) and *L. terrestris* needs sufficient soil moisture to survive. The presence of abundant *L. terrestris* populations on very sandy soil raises the following question: *was this soil moist enough to host* L. terrestris*, despite being sandy, or, was* L. terrestris *able to live here, despite the soil being prone to drought*? Both may be true.

This question cannot be fully answered using the available data, but future research could address it by assessing the relationship between **burrow depth** and soil moisture at various depths. In a previous laboratory study, the burrow depth of *L. terrestris* increased when temperature was higher and soil moisture was lower (Perreault & Whalen, 2006a), indicating that the earthworms adjust their burrow depth in response to soil moisture conditions. In a field study, flexibility in burrowing behaviour was also observed under humid conditions; the authors pose that *L. terrestris* can survive in shallow soils in a humid climate with no severe freezing of the topsoil (Butt & Nuutinen, 2024). In the present study, it was observed that *L. terrestris* burrowed to > 60 cm depth after inoculation (Chapters 4 and 5). In future experiments, it would be useful to measure soil moisture at various depths and test for a relationship between soil depth, soil moisture content and *L. terrestris* survival. In this way, it could be confirmed whether adjusted burrow depth is an effective survival strategy in habitats that are prone to drought, such as sandy soils.

3.3 Mesocosms: their merits and their disadvantages

The setup used for the experiments was that of in-field mesocosms (Chapters 3, 4 and 5). In-field mesocosms offer the advantage of approaching agricultural reality more closely than a lab environment, while providing a level of control and manageability that is higher than in an open-field study. The setup we used lent itself to experimenting at a larger scale than would have been possible in the lab, and this helped us to explore the potential for inoculation as a workable management practice. Mesocosms play an important role in bridging the gap between the lab and the field, and they allowed us to study the survival and reproduction rate of all the introduced *L. terrestris*. In this phase of *L. terrestris* inoculation research, where the main aim was to test whether the species would actually survive and reproduce in sandy soils with and without a resident population, mesocosms were very effective. They offered realistic soil conditions in many aspects (such as profile, biotic community, structure and weather conditions), they enclosed the inoculum without limiting burrowing depth and they allowed for easy replication. Yet, mesocosms have their disadvantages too. Below, I discuss three possible drawbacks of the mesocosm setup and how they may interfere with the earthworm environment in undesired ways. I end with some suggestions for addressing these challenges.

First, the diameter of the mesocosms was limited (61 cm), thus inhibiting the earthworms' freedom of movement. In lab studies, *L. terrestris* has been kept in much

smaller confinements while still surviving and reproducing (Butt, 2011c; Butt et al., 1992; Perreault & Whalen, 2006b), and therefore I do not consider the diameter of the mesocosms too small to test whether *L. terrestris* would be able to survive and produce offspring. Nevertheless, if inoculation is to be upscaled in the future, a larger test setup would be necessary to assess inoculation under circumstances where *L. terrestris* can display its natural behaviour even better, as the species is known to be able to roam several metres in one night (Mather & Christensen, 1988). The restriction of the ø 61 cm mesocosm could prevent the earthworms from finding a preferred location for a burrow or impose sub-optimal mating choices. It was found in previous research that earthworms show rigorous mate selection (Domínguez & Velando, 2013).

Second, the mesocosms were made of steel, which is a good heat conductor. Therefore, the soil near the steel of the mesocosms may have heated and cooled more quickly and to higher and lower temperatures than the surrounding soil. Moreover, the mesocosm wall and protective nets decreased air movement and might have caused a greenhouse effect to some degree. Soil temperature is well known to influence earthworm activity and development (Berry & Jordan, 2001; Perreault & Whalen, 2006b). If the mesocosms indeed warmed up the soil, this may have increased earthworm activity in the vicinity of the mesocosm wall at moments when the surrounding soil was still too cold, for example on mild days in early spring. This may have stimulated earthworm growth and development. On very hot days, there may have been an increased risk of the soil becoming too hot for earthworms. A soil temperature of 30°C has been found to be lethal for *L. terrestris* after 14 days, whereas 20°C is ideal for mass rearing (Berry & Jordan, 2001). However, this is speculative, as soil temperature in the mesocosms was not measured. I recommend monitoring soil temperature in future research for a more complete overview of the experimental conditions in general, and also to either confirm or rule out the effect of steel mesocosms on the soil temperature.

Lastly, vegetation development within the mesocosms sometimes differed from the surrounding grassland, due to an altered microclimate. Dominance of grass versus clover or the presence of dandelions (*Taraxacum officinalis*) in some mesocosms was likely to have had some effect on the earthworm population, as vegetation type largely determines the quality and quantity of litter input, which is an important food source for earthworms. Previous studies have demonstrated that botanical composition can alter earthworm populations (Fischer et al., 2014). It is difficult to prevent

this in in-field mesocosms; therefore, the effect of variation between mesocosms on the data was mitigated by using replicates.

I have some suggestions to meet the aforementioned challenges and to move the field forward. Larger mesocosms, also referred to as **macrocosms**, or an **open-field setting** could overcome the size-related limitations of the ø 61 cm mesocosms. A setup like this would allow *L. terrestris* more freedom of movement and mate choice. Additionally, it could decrease the risk of deviant vegetation inside the mesocosms. A trade-off would be that protection against predators by using a net is more difficult and it would no longer be feasible to hand-sort all the soil. This was still possible with the ø 61 cm mesocosms that were used in Chapters 3, 4 and 5, thus helping us to avoid the risk of overlooking heterogeneity *within* mesocosms. To limit the effect of variation *between* mesocosms we used 3-4 replicates per treatment. More **replicates** could further mitigate the effect, but would also significantly increase the workload. Alternatively, **weeding** could be applied to gain more control over the botanical composition within the mesocosm. Overall, mesocosms worked well in this phase of the research. The size of the mesocosms we used was suitable for exploring the potential of *L. terrestris* inoculation at a 'proof of principle' level. Now that the results of the mesocosm inoculation experiments (Chapters 4 and 5) have shown potential for *L. terrestris* inoculation, open-field inoculation experiments could be conducted to test the feasibility and workability at a larger scale.

4. Practical implications and remaining questions

The research discussed in this thesis addresses harnessing the qualities of earthworms, particularly of deep-burrowing *L. terrestris*, as an ecological innovation to improve climate adaptation. I deliberately chose to focus on gathering field data from operating dairy farms. The aim of this research was not only to generate scientifically underpinned knowledge, but also to work towards action perspectives for farmers and policymakers who are looking for new prospects on water regulation. The following section presents some practical implications and remaining questions with regard to three different stakeholders: farmers, water board policymakers and earthworm breeders. This section does not intend to provide an exhaustive list of all possible interventions and their effect on earthworms, it is limited to the action perspectives that follow from the research in this thesis.

4.1 Grassland management and *L. terrestris* inoculation: Action perspectives for farmers

4.1.1 Earthworm education and monitoring

The inventory described in Chapter 2 shows that *L. terrestris* is already present in many grasslands on sandy soil in the south of the Netherlands (60% of the sampled grasslands in the research area). A first step for farmers interested in learning more about the earthworm population in a field would be to become acquainted with simple in-field earthworm quantification methods and with knowledge on how and when to apply these. One such method could be hand-sorting a soil square of known dimensions, preferably combined with mustard extraction. Such knowledge would enable farmers to quantify and monitor the earthworm population in their soils. Subsequently, farmers should acquaint themselves with *L. terrestris*, so that they can recognise the species, its middens and its burrows in the field. Study groups could facilitate this; there is already a reliable field-guide for earthworm species in the Netherlands (Krediet & Kimpel, *De Nederlandse Regenwormen*, 2024), and species identification apps are also capable of recognising *L. terrestris*. If they can quantify earthworms and recognise *L. terrestris*, farmers would be able to check the state of the earthworm populations in their fields.

4.1.2 Adjusted grassland renewal strategies

Chapter 3 clearly demonstrated that permanent grassland yielded the highest earthworm abundance. Grassland renewal strategies include tillage (soil disturbance) and conversion to arable land (prolonged loss of vegetation cover); both had a negative effect on earthworm abundance six months after application. The combined effect of tillage and vegetation loss had the greatest negative effect. Chapters 4 and 5 demonstrated that earthworm populations are sometimes capable of recovering in 8-12 months if food is abundant (Figure 6.1A, 6.1B, 6.1D and 6.1E), but that this is not always the case (Figure 6.1C and 6.1E). A practical recommendation would be to minimise grassland renewal and only apply it if the benefits are expected to outweigh the costs. If grassland renewal is applied, the sward should be incorporated into the soil to facilitate a quick recovery of the earthworm population. Reseeding without temporal conversion to arable land is advised, as arable crops generally provide less litter for earthworms to feed on for a prolonged period of time. High-quality food for earthworms could be provided by introducing legumes such as clover or alfalfa and applying organic fertilisers.

Another valuable lesson learnt from this research was that soil texture and the weather can have a large impact on earthworm abundance. It is important that farmers recognise that there are not only preconditions that can be influenced (such as soil disturbance, botanical composition and fertiliser) but also preconditions that cannot be changed (such as soil texture, weather and predation). It is important to invest in the quality of manageable preconditions, so that if the preconditions outside the sphere of influence are also met, earthworm populations will thrive and grassland quality will benefit from this.

4.1.3 *L. terrestris* inoculation

This thesis shows that inoculation of *L. terrestris* could be a way to establish an *L. terrestris* population in a grassland which is currently devoid of the species. Thus far, this has only been tested in mesocosms and in limited time frames (15 months maximum). Therefore, it is too early to say that *L. terrestris* inoculation could be recommended from a scientific perspective. Additionally, the mortality rate we observed was high, 74-94% in 12 and 15 months, respectively. Before applying inoculation on a larger scale, further experiments would preferably result in improved inoculation strategies with smaller losses, which is desirable for both ethical and financial reasons. To make this happen, it is crucial that scientists and farmers co-operate. Farmers should indicate the challenges that they face when trying to create the right conditions for *L. terrestris*, and scientists should use experimental setups that also answer practical questions. Such questions include at which density should *L. terrestris* be applied, what *L. terrestris* origin is preferable, at what life stage should the inoculum be, which time of the day, season and rotation would be preferable for inoculation, and how can the earthworms be spread on the field. Research should be conducted mostly in the field.

4.2 Recommendations for water boards

Water boards face the challenge of providing enough water of the right quality to meet the needs of the drinking water supply, agriculture, nature and recreation. Changing precipitation patterns and diverging needs make this an increasingly complex task, especially on the elevated sandy soils in the south and east of the Netherlands, as these soils are prone to drought and it is difficult to draw water to these higher grounds. Additionally, as a result of their elevated position, water that does not infiltrate can run off, increasing the risk of floods in lower-lying areas. Hence, water infiltration and retention are crucial on elevated sandy soils. Enhancing the

water infiltration rate and capacity in grasslands would lower the pressure on water boards as it would lead to increased drought resistance and more self-sufficiency in water supply. Harnessing the qualities of earthworms does not offer a simple solution to the complex matter of climate adaptation, but it could be part of an urgently needed systemic change towards land management that is better aligned with future climate conditions.

In addition, water boards are in the unique position that they connect multiple domains as they operate in close contact with provincial governments, farmers and research institutes. Moreover, they have the financial means to drive innovations, for example through incentive schemes and research funds. Consequently, water boards can play an important role in bridging gap between science and practice that was mentioned above. They support the development of ecological innovations for improved water regulation, such as the research described in this thesis. Based on the work we did, I conclude that earthworm management and inoculation has definite potential as a management practice, given the pre-existing knowledge about earthworm burrows as enhancers of water infiltration and the advancing insights from this work into the suitability of grasslands on sandy soil to host *L. terrestris* and the ability of *L. terrestris* to persist and reproduce after inoculation into these grasslands. However, long-term and open-field studies are necessary to determine what its contribution could mean in practice. Importantly, possible trade-offs should be investigated. These entail a potential rise of greenhouse gas emissions from the soil with greater earthworm abundance and elevated nutrient leaching when water infiltration increases, which could negatively affect water quality. The latter is important for waterboards and especially relevant sandy soil, which is more prone to nutrient leaching compared to fine textured soils (Shepherd & Bennett, 1998).

Finally, water boards can promote further progress of earthworm management for improved water regulation by bringing together their own expertise, the farmers that operate in their region, and the research institutions, so that these can work jointly on strategies that meet everyone's needs and that can be realised in practice. In the case of *L. terrestris* inoculation, farmers could be informed about the added value of earthworms and particularly anecic species ("*Regenwormen op het melkveebedrijf*", Louis Bolk Instituut, 2014; "*Wormen als multifunctionele werkers in je bodem*", Louis Bolk Instituut, 2026). They could then be invited to participate and share their knowledge in accessible demo projects where *L. terrestris* is inoculated in on-farm open field settings. These demos should be combined with grassland management

measures that support *L. terrestris* survival and reproduction, such as permanent grassland, grass clover mixtures and organic fertilisers. Monitored by researchers, such a project could offer a broad-based perspective for practically feasible and scientifically-backed ecological innovation.

4.3 The potential role of *L. terrestris* breeding

Currently, *L. terrestris* breeding is still in its infancy. The species can be purchased, but at the moment of writing the commercially available *L. terrestris* are not bred in captivity. Instead, these individuals are collected from fields in Canada. The global supply of 700 million individuals comes from Southwestern Ontario, where the species reaches population densities of beyond 300 ind. m^{-2} (Steckley, 2020). Farmers with fields that are home to abundant *L. terrestris* populations rent out their land to worm-pickers. Earthworms are collected at night by simply picking them up from the ground as they surface, a task mostly carried out by Vietnamese immigrant workers. Steckley conducted two detailed studies on the worm-picking industry (Steckley, 2022; Steckley, 2020).

If inoculation of grasslands with *L. terrestris* was to become an agricultural practice, it would involve a great deal of shipping, energy and labour. However, many of these costs could be avoided if *L. terrestris* was bred locally. In the past few years, several Dutch earthworm breeders have been developing a breeding strategy for *L. terrestris*, but this has proved to be rather difficult (personal communication with Megrow and WormsSystems). The challenge of breeding *L. terrestris* has also received scientific attention; it was mostly the dedication of Butt and co-workers that has provided a body of research on culture techniques and optimisation (Butt, 1991; Butt, 2011a; Butt, 2011b; Butt, 2011c; Butt et al., 1992b, 1994, 1997; Lowe et al., 2023; Lowe & Butt, 2005). However, *L. terrestris* is not the only anecic earthworm in the Netherlands, as both *A. longa* and *Aporrectodea nocturna* also occur in Dutch grasslands and create deep burrows. Although these species are less common, it may be of interest to explore the suitability of these species for breeding and inoculation. Several researchers outside the Netherlands have reported positive results regarding inoculation of *A. longa* (Butt et al., 1993; Schon et al., 2016).

Currently, communication between practice and science seems to fall short, which is regrettable. Both fields would be able move forward if we were to combine the valuable insights from research and a systematic approach on the one hand, and the incredible amount of hands-on experience and practical knowledge among

earthworm breeders and vermicomposters on the other. Communication is key here, and research could focus more on practical applications; if earthworm breeders express their needs and questions, their breeding practices could be guided by scientific insights into *L. terrestris* production systems. There seem to be two plausible pathways along which *L. terrestris* mass rearing could develop: indoor culturing in a semi-controlled environment and open-field site-specific stimulation. It is beyond the scope of this thesis to work out a detailed description of what these systems could look like, but I will provide a bullet point list of some requirements I think should be met. Please note that these lists are not intended to be exhaustive.

4.3.1 Indoor culturing

Soils that are devoid of *L. terrestris* but that seem suitable to host a population could be inoculated with the species. The inoculum could be cultured by earthworm breeders, who currently produce mainly *Eisenia fetida* and *Dendrobaena veneta*. Given their network, experience and knowledge about earthworms, it seems logical that these breeders could also use their facilities for *L. terrestris* culturing. Indoor culturing offers various benefits compared to worm picking in the field, such as a more controllable environment (for example moisture, temperature, resource input and light regime can be fine-tuned), avoidance of laborious worm-picking, and virtually no risk of predation. The following factors should be kept in mind to make this feasible:

- A system could be designed in which earthworms feed on organic waste and turn this into soil amendments. In this way, earthworm breeding can contribute to the circularity of the food system, and waste processing would offer possibilities for revenues apart from selling earthworms.
- The price of the earthworms should be kept low (for example by feeding on waste and selling vermicompost) as large quantities would be needed for agronomic application. Currently *L. terrestris* is commercially available in the Netherlands for approximately €70 kg^{-1}. Hypothetically, to apply 20 gr m^{-2} on 1 ha would cost €14,000, excluding the labour necessary for inoculation. This is an unrealistically high investment for a farmer. For comparison, drainage including materials and instalment costs €1,000-3,000 ha^{-1} in the Netherlands. Earthworms could contribute to more ecosystem services than drainage systems, but not enough to make up for an investment of this order of magnitude on agricultural soil.

- Breeders should offer adequate guidance for the inoculation process to ensure the highest chances of successful establishment. This would encompass for example timing (e.g. what part of the day, at which point in the growing season, at what time of the year and at which moment in crop rotation), inoculation method and aftercare (e.g. soil cultivation, application of organic input and pesticide use). Researchers should co-operate with breeders and farmers to work out the best practices.

4.3.2 Site-specific stimulation

If a resident population of *L. terrestris* is present and there is a desire to increase its abundance, stimulation of the species would be preferable to inoculation. Stimulation avoids the inoculum having to adjust to a new environment as well as the mortality rate that is often linked to that. In addition, in normal inoculation there is a moment where *L. terrestris* lies on top of the soil, and this moment is now avoided, decreasing the risks of predation and desiccation.

- Optimal management from the perspective of *L. terrestris*, and optimal management from the perspective of the farmer should be weighed up, especially in terms of crop rotation/botanical composition, input of organic matter and soil cultivation. Compromises need to be found for the conflicting interests of the farmer and the earthworm. Examples include the addition of clover to grassland, the application of organic fertilisers such as farm yard manure instead of artificial fertilisers, no tillage, or at least no inversion-tillage, and no root crops.
- A simple and standardised monitoring method should be offered to farmers, such as hand-sorting soil squares combined with mustard extraction, or, in the future, one of the methods proposed in section 3.1. This would enable farmers to adjust their management practices to developments in the field.
- The practices to be carried out by farmers should also be workable for them, so they should not be time consuming and no special equipment should be necessary.
- Farmers should have access to information – also from non-commercial parties – about earthworms and earthworm inoculation or stimulation. Research institutes and agronomic education facilities play an important role in this.

5. Concluding remarks

Even though climate change *mitigation* should be priority number one, climate adaptation is an absolute necessity, as the consequences of climate change are already ongoing and will continue into the future. Climate change and the resulting weather extremes put pressure on the functioning of ecosystems. This negatively affects society as it interferes with food safety, flood risks, stable revenue models for farms, drinking water supplies and biodiversity. To safeguard the continuation of the ecosystem services on which humanity relies, the robustness of natural and agricultural ecosystems should be reinforced. We should look for climate adaptive measures that are embedded in the ecosystem in which they are applied; such measures are also referred to as *nature-based solutions* or *ecological innovations*.

An example of such a nature-based solution or ecological innovation is the acknowledgement and stimulation of the positive impact of the deep vertical burrows of anecic earthworms such as *L. terrestris*. This entails making use of the unique qualities of a component that is already – quite literally – embedded in the ecosystem in which it operates, namely the positive effect of earthworm burrows on the water infiltration rate and water-regulating capacity of the soil. Even if anecic earthworms have been inoculated into the soil, they can only develop a stable population if the conditions are right, and this emphasises that they are not just a simple external input used to solve a simple problem but rather an integral part of the grassland ecosystem and its functioning.

The aim of this thesis was to improve our understanding of the current and potential role that earthworms and particularly *L. terrestris* can play in improving the water-regulating capacity of grasslands on sandy soil. We found that populations of the species exist in grasslands on sandy soil, sometimes even in high densities. Our study revealed that the presence and abundance of *L. terrestris* positively correlates with the soil's loam content. However, the exact factors that determine the presence and abundance of *L. terrestris* remain difficult to identify. We also found that it is possible for both commercially purchased and locally collected *L. terrestris* to survive for at least 15 months, produce offspring and burrow > 60 cm deep after inoculation into a mesocosm, even though mortality rates were 74-96%. The experiments also demonstrated that grassland renewal strategies which include tillage and loss of vegetation cover can result in significantly reduced earthworm populations six months after application. Under favourable conditions, earthworm populations,

including *L. terrestris*, demonstrated full recovery of the population within one year after severe disturbance.

The practical implications of these findings are discussed more extensively in the previous section. The next step would be to explore whether and how successfully *L. terrestris* inoculation could take place in long-term open-field settings. Potential trade-offs such as increased nutrient leaching and greenhouse gas emissions should be addressed in future research in order to prevent undesirable side-effects of large-scale application. Farmers should learn more about earthworms and, if possible, adjust their management to support or stimulate their populations. If *L. terrestris* is absent, inoculation of the species could be considered. Earthworm breeders could locally breed *L. terrestris* for inoculation purposes and work out practical strategies for successful inoculation at a reasonable price. Water boards could use their network to spread and develop expertise regarding the important role of earthworms in the water-regulating capacity of grasslands. Furthermore, they should financially incentivise initiatives that could move this ecological innovation from theory to practice. With the joint endeavour of researchers, farmers, earthworm breeders and policy makers, the qualities of earthworms can support climate adaptation.

Wageningen Worm Charming Championship 2024, "The Blood Worms"



Wageningen Worm Charming Championship 2025, “PFAS, praat erover”



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Appendices

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thesis-studenten, externe krachten, familieleden etc., die dagenlang geholpen hebben om de gigantische hoeveelheid werk te verzetten die nodig was om alle grond uit de buizenproeven te doorzoeken op wormen: ontzettend ontzettend ontzettend bedankt. Jullie leuke gesprekken, hulp bij het vastklemmen van een wegwaaiende partytent, koekjes, playlist-toevoegingen, flexibiliteit en harde werk waren fantastisch. Dan de collega's van het lab, met name Jan Verkampen en Riekje Bruinenberg hebben vele uren doorgebracht achter een binoculair en in de dampen van wormenmonsters op alcohol. Hun toewijding en vakkundigheid heeft ertoe geleid dat we goede data hadden en zelfs soortspecifieke informatie over juveniele regenwormen, een grootse prestatie, dankjulliewel. Bij het analyseren van de data was de hulp van Joachim Deru, Nyncke Hoekstra, Pedro Janssen en Jonas Schepens onmisbaar, heel erg fijn dat jullie me daarin wilden ondersteunen (en dat jullie het nooit lieten merken wanneer ik een domme vraag stelde). Pedro verdient nog een speciale vermelding in de ondersteuning, omdat onze wedstrijd om de eerste te zijn die onder Nick promoveerde een goede motivatie was om de vaart erin te houden. Bedankt ook aan Maaike van Agtmaal en Nick Quist voor het geven van waardevolle feedback op mijn manuscripten. Lyanne van den Berg wil ik bedanken voor haar morele steun in onze gesprekken over waarom je in 's hemelsnaam eigenlijk een PhD zou willen doen.

Een van de mooiste aspecten van deze PhD was dat ik in de échte wereld aan een écht probleem mocht werken. Daar hoorden ook échte wormen en échte boeren bij. Dankjewel Gerrit Verhoeve, Jan-Dirk van de Voort, Evert Kremer, Manon van Balkom, Jo van Balkom, Guus van Roessel en Cees van Roessel en alle (Duin)boeren die hebben meegewerkt aan de inventarisatie. Geweldig dat we op jullie percelen veldexperimenten mochten aanleggen, bedankt ook voor het meedenken daarbij, de verwarmde kantines tijdens gure veldwerkdagen en de uitwisseling over het onderwerp. Moge het jullie én jullie wormen goed gaan.

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fotograaf Koen Verheijden – die de worm een warm hart toedraagt – zeer erkentelijk. De fenomenale tekeningen voorafgaand aan de samenvatting en de appendices zijn van de hand van Donna Algra, heel erg bedankt voor deze wilde kunstwerken!

Ik heb altijd veel dingen naast mijn PhD gedaan, ik ben zelfs tot de hypothese gekomen dat het hebben van meerdere banen wel eens zou kunnen helpen om je proefschrift sneller af te hebben. Het heeft mij in ieder geval geholpen om ruimschoots voldoende afwisseling te behouden en daardoor heb ik relatief weinig tijd hoeven besteden aan uit het raam kijken zonder een steek verder te komen. Graag wil ik daarom ook nog even de schijnwerper richten op NatuurlijkSportief, De Vuurplaats en alle opdrachtgevers die mij in de afgelopen jaren op allerlei manieren hebben uitgedaagd om iets betekenisvols te doen. Bedankt Teun van der Klis, voor het oprichten van NatuurlijkSportief en dat je mij een locatie hebt toevertrouwd, bedankt ook aan alle andere buitenspeelbazen voor wat jullie uitdragen, want er wordt nog lang niet genoeg buitengespeeld. NatuurlijkSportief geeft mijn innerlijke rouwdouwer en al mijn sportieve speelsheid kredietwaardigheid. Een hele grote gore modderige dankjewel ook aan alle sporters van NatuurlijkSportief Utrecht. Bij jullie kon ik mijn creativiteit kwijt, kon ik zo nu en dan mijn frustratie omzetten in worstelpartijen, mocht ik altijd vertellen over wormen en dankzij jullie medewerking heb ik ook tijdens de lange periode van schrijven het eelt op mijn handen en de schrammen op mijn benen kunnen behouden. Ik hoop dat we nog heel veel samen gaan rauzen. Dankjewel aan alle (oud-)collega's van De Vuurplaats met wie ik zo'n mooie tijd heb op het landgoed. Buiten koken is een geweldige tegenhanger van onderzoek doen (snel resultaat én complimentjes, wat wil je nog meer?), ook de onderlinge sfeer, de geweldig mooie omgeving en het praktisch samenwerken brengen me veel plezier, dankjulliewel daarvoor.

Dan mijn (oud-)huisgenoten, waar ik ontzettend blij mee ben, mijn sportmaatjes van Trajectum met wie ik graag in de touwen hang en vooral ook: mijn fantastische vrienden. Ik ga geen namen noemen, om mezelf het risico iemand te vergeten te besparen, maar dat maakt me niet minder dankbaar voor alle steun, interesse en afleiding die jullie me geboden hebben in de afgelopen jaren. Dankzij jullie bestond mijn leven niet alleen uit gaten graven, wormen tellen en typen, maar ook uit avonturen in de bergen, dansen, goede gesprekken, (winter)zwemmen, samen eten, kampvuurtjes, verkleedpartijen, trailruns, (wild)kamperen, ijsjes eten, samen filosoferen, afzien tijdens survivalruns, filmavonden en ga zo maar door. Bedankt voor alles tot nu toe en ik hoop dat we in de toekomst nog heel veel meer gedeelde herinneringen gaan maken.

Natuurlijk wil ik ook graag mijn familie bedanken. Opgroeien in een bonte verzameling van mensen heeft me de vaardigheden gegeven waar ik nu nog elke dag de vruchten van pluk. Pap en mam, ik ben ontzettend trots op jullie en er is geen beginnen aan om te benoemen wat ik allemaal aan jullie te danken heb. Toch wil ik twee specifieke levenslessen noemen waar ik veel aan gehad heb tijdens dit proces. De eerste is "*als wat je zoekt er nog niet is, dan maak je het toch gewoon zelf*"; die instelling heeft me altijd geholpen heeft om oplossingsgericht te werken. De tweede is "*zet je er maar overheen*", een PhD doen is namelijk niet altijd leuk, maar deze les heeft me in staat gesteld om door te gaan bij tegenslag. Bedankt voor wat jullie me hebben meegegeven. Bedankt ook aan Sjef die mij mijn hele leven lang al laat zien dat mens-zijn vele vormen aan kan nemen. Je helpt me mijn eigen pad te bewandelen en ik ben een bofkont dat ik jouw zusje ben. Suz en Mart, ik prijs mezelf telkens weer gelukkig met jullie, we kunnen samen praten, lachen, stil zijn en op elkaar leunen wanneer nodig. Jullie hebben me door het laatste deel heen gesleept, heel erg bedankt daarvoor. "Thuis" in Limburg stonden mijn maatjes Lola 🐾 en Flip 🎧 altijd klaar voor een knuffel en een wandeling, dankjulliewel voor jullie kameraadschap.

Ik realiseer me heel goed dat ik een enorme geluksvogel ben met zoveel geweldige mensen om me heen, vandaar dat de lengte van dit dankwoord intussen volledig de spuigaten uitloopt. Als allerlaatste wil ik nog graag een algemeen gevoel van dankbaarheid uitspreken. Wat mooi dat ik dit allemaal mee mag maken!

About the author



Roos Christina Frits van de Logt was born on the 21st of February in 1994. She grew up in the Dutch countryside in the province of Limburg, where she spent most of her time looking after all kinds of animals, riding horses, climbing trees, conducting culinary experiments in her parents' kitchen, and reading animal encyclopaedias as well as any other book she could lay her hands on. As a child, her curiosity and a certain sense of morality often sparked questions about the how and why of the world. Sustainability and vegetarianism became important topics for her from a young age.

Roos always had a broad field of interests, expressed in a never-ending list of hobbies and a great variety of side jobs. After high school, she took a gap year to spend time on all these things that had always had to compete with school and homework. She also seized the opportunity to travel to Ecuador to learn Spanish and do volunteer work. After that, no one was surprised when she decided to study Biology at Wageningen University.

While studying, she set up a small pop-up restaurant concept, aiming to seduce omnivores into herbivory by surprising them with tasty vegetarian food. Around the same time, she discovered a passion for survivalrunning and became one of the founders (and a trainer) of WOEST, the student survivalrun association in Wageningen.

Always having enjoyed being taught by student assistants, she decided to become one hersel. Over the years, she assisted in numerous courses, where she discovered how much she enjoyed teaching practicals and leading field excursions. In between her BSc and MSc, she took another gap year and travelled to Peru and Colombia to improve her Spanish, while also falling in love with rock climbing.

Her specialization was bio-interactions, and during her MSc she took a number of courses related to organic farming. Her thesis gave her the opportunity to spend time in the Mexican rainforest, where she studied the functional traits of fine roots in the context of secondary succession. Over the course of her MSc, her relationship with science was one of both love and hate.

Nevertheless, the overlap between biology, sustainability and food production systems turned out to be a sweet spot for her. Right in the middle of this sweet spot, she encountered the Louis Bolk Institute and, when she dug a little deeper, she found earthworms.

Roos was happy to have the opportunity to persue an external PhD while working a researcher. She has been dividing her time between earthworm research and several other projects related to soil health and transition in the food system. To maintain her sanity and also make room for her other fields of interest, she works part-time at the Louis Bolk Institute and is part-time self-employed. She offers playful outdoor sports training (she is affiliated with NatuurlijkSportief), cooks with and for people over an open fire, and is involved in outdoor and nature education for both children and adults.

List of publications

Scientific papers

van de Logt, R., van der Sluijs, T., & van Eekeren, N. (2023a). Lumbricus terrestris abundance in grasslands on sandy soils in relation to soil texture, hydrology and earthworm community. *European Journal of Soil Biology*, *119*, 103545.

van de Logt, R., Versteeg, C., Struyk, P., & van Eekeren, N. (2023b). The anecic earthworm Lumbricus terrestris can persist after introduction into permanent grassland on sandy soil. *European Journal of Soil Biology*, *119*, 103536.

van de Logt, R., & van Eekeren, N. (2025). Factors affecting colonisation success of the anecic earthworm Lumbricus terrestris (L.) in mesocosms on temperate pasture. *Plant and Soil*, *513*(2), 2621-2637.

Roca Vallejo, R., Krzywoszynska, A., Katikas, L., Naciph Mora, K., Husseini, M., Rodrigues, S. M., **van de Logt, R.**, Johnson. K., Vrščaj, B., Ramezzano, C., Črnec, K., & Ballstaedt, A. (2025). Assessment of knowledge gaps related to soil literacy. *Land*, *14*(7), 1372.

Roos van de Logt is also (co-)author of non-scientific publications. For a current overview of publications, see https://www.louisbolk.nl/over-ons/medewerkers/roos-van-de-logt.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 30 ECTS (= 22 weeks of activities)

Review/project proposal

- Management and inoculation strategies to promote anecic earthworm species *Lumbricus terrestris* (L.) in agricultural grasslands. | 4.5 ECTS

In-depth / Topical / On-site Postgraduate Courses

- Root Ecology | WGS | 2025 | 1.5 ECTS
- Plant sap analysis course | NovaCropControl | 2025 | 0.1 ECTS
- On-site compost tea workshop and discussion | LBI & BioRomeo | 2025 | 0.1 ECTS
- International symposium on earthworm ecology | WUR, Louis Bolk Institute, VU | 2026 | 2.5 ECTS

Deficiency, refresh, brush-up courses

- Slim Landgebruik | WUR & Louis Bolk Instituut | 2020-2022 | 1.5 ECTS
- Basic statistics | PE&RC | 2025 | 1.5 ECTS

Laboratory training and working visits

- Soil assessment course | Louis Bolk Institute & Coen ter Berg | 2021 | 0.3 ECTS
- eDNA | Leiden University of applied sciences (2023), Datura (2024) | 0.6 ECTS
- Plant sap measurements | NovaCropControl | 2025 | 0.2 ECTS
- On-site earthworm species determination | Louis Bolk Instituut & Anne Krediet | 2026 | 0.3 ECTS

Invited review of journal manuscripts

- Agriculture, Ecosystems & Environment | Soil moisture and fertility drive earthworm diversity in north temperate semi-natural grasslands | 2023 | 1.0 ECTS

- Geoderma | Intensive culture of anecic earthworms (*Amynthas aspergillum*) induces soil organic carbon accumulation on a vertical scale by regulating microbial biomass and community structure in South China (title at the moment of reviewing) | 2025 | 1.0 ECTS

Competence, skills and career-oriented activities

- Scientific writing (course) | UU | 2024 | 0.9 ECTS
- Nature Inclusive Teaching | WUR Teaching and Learning Centre | 2025 | 0.9 ECTS
- The Choice: action-focused personal leadership training | WGS | 2026 | 0.7 ECTS
- Career talk: what to do after your PhD? | PPC | 2026 | 0.1 ECTS

Scientific Integrity/Ethics in science activities

- Scientific Integrity | WGS | 2025 | 0.6 ECTS

PE&RC Retreat, PE&RC Day, and other PE&RC events

- PE&RC Mid-term retreat | 2025 | 0.6 ECTS
- PE&RC day | 2025, 2026| 0.6 ECTS

National/local scientific meetings, seminars, and discussion groups

- Kick-off meeting National Centre for Soil Ecology | 2023 | 0.3 ECTS
- Nationale Bodemtop | 2024 | 0.3 ECTS
- Caring congress | 2024 + 2025 | 0.6 ECTS
- Deltaplan veehouderij Awards | 2023 | 0.3 ECTS
- Biobeurs | 2024 | 0.3 ECTS
- Bio velddag | 2024 | 0.3 ECTS
- 100 Jaar BD conferentie | 2024 | 0.3 ECTS
- International Symposium on Earthworm Ecology ISEE (Rennes, France) | 2022 | 1.5 ECTS
- Netherlands Annual Ecology Meeting (presentation) | 2025 | 1.3 ECTS
- Louis Bolk Institute “lunchlezingen” | 2021-2025 | 5.1 ECTS
- Organisation ISEE 2026 | 2025-2026 | 1.0 ECTS
- Bodemkennisdag | 2025 | 0.3 ECTS
- BodemUP on tour | 2025 | 0.3 ECTS
- Presentation at international Legume Legacy PhD meeting | 2026 | 0.3 ECTS

International symposia, workshops and conferences

- EGF General Meeting (2 posters) | Leeuwarden | 2024 | 2.5 ECTS
- Wageningen Soil Conference (workshop) | Wageningen | 2024 | 0.3 ECTS
- Symposium National Centre for Soil Ecology (presentation) | Nijmegen | 2024 | 1.3 ECTS

Societally relevant exposure

- Newspaper article (Volkskrant) | 2020 | 0.1 ECTS
- Radio item (vroege vogels) | 2020 | 0.1 ECTS
- Newspaper article (Trouw) | 2024 | 0.1 ECTS
- Radio interview (radio1 Belgium) | 2024 | 0.1 ECTS
- WUR Biodiversity Challenge earthworm excursion | 2023 | 0.3 ECTS
- Presentation at Bio-boerendag Brabant | 2024 | 0.3 ECTS
- Workshop at LTO farmers gathering | 2024 | 0.2 ECTS
- Earthworm excursions with kindergarten | 2023-2026 | 0.3 ECTS
- Presentation at Duinboeren meet-up | 2021 | 0.1 ECTS
- Article in Bodem | 2024 | 0.2 ECTS
- Article in V-focus | 2024 | 0.2 ECTS
- Poster presentation at LBI Relations Day | 2025 | 0.1 ECTS
- Article in Ekoland | 2025 | 0.1 ECTS
- Article & presentation for VvM (Vereniging van Milieuprofessionals) | 2025 | 0.3 ECTS
- Collaboration in art project Next Nature | 2025 | 0.1 ECTS
- Article in H2O | 2026 | 0.2 ECTS
- Earthworm workshop with Toekomstboeren study group | 2026 | 0.3 ECTS
- Earthworm workshops at Louis Bolk Instituut anniversary | 2026 | 0.3 ECTS

Lecturing/Supervision of practicals/tutorials

- FSE course Analysis and design of organic farming systems | 2025 | 7 days | 2.1 ECTS
- Field practical at MBO Boxtel | 2026 | 1 days | 0.3 ECTS

BSc/MSc thesis supervision

- Research topic 1: Inventory of anecic earthworms on sandy soil | 0.9 ECTS

A

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