



## Soil moisture and fertility drive earthworm diversity in north temperate semi-natural grasslands

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### ABSTRACT

Intensive management of arable land reduces earthworm density and diversity. This may impair earthworm-mediated soil functions, such as nutrient mineralization and soil structure formation. To sustain earthworm source populations for re-colonization of cultivated soils, it is therefore important to preserve habitats with high earthworm diversity. Semi-natural grasslands, with a long continuity without soil disturbance, could serve as such earthworm diversity reservoirs. This is particularly important in mixed agricultural landscapes with elements of multiple land uses. Nonetheless, earthworm density and diversity vary greatly among grasslands. To preserve and optimally manage the most suitable grasslands, knowledge about which grassland characteristics best explain earthworm diversity is needed. Additionally, we have a limited picture of earthworm diversity in general, because previous studies have neglected juvenile earthworms and cryptic species. The juvenile fraction commonly comprises the main part of earthworm samples, whereas morphologically inseparable cryptic species account for an unknown fraction. This fraction is of particular importance, as juveniles reflect the local reproductive and regeneration potential of earthworm populations and communities. To determine the full species composition of earthworm communities, we sampled earthworms from 28 semi-natural grasslands in south-central Sweden and identified them to species by DNA barcoding. To test how grassland characteristics explain earthworm density, diversity, and community composition, we measured several characteristics of soils, vegetation, and management of the grasslands, and descriptors of the surrounding landscape. DNA barcoding revealed nearly twice as many species as were identified morphologically. Earthworm densities were higher in grasslands with higher Ellenberg moisture indicator values and lower soil C:N ratios. The diversity and occurrence of many earthworm species was also higher in grasslands with higher soil moisture indicator values and lower C:N ratios, and further increased with habitat heterogeneity. Certain species occurred more likely in grasslands with higher grazing intensity. Epigeic earthworms, which live in and feed on surface litter, were more common in grasslands with higher moisture indicator values and SOM content. Thus, dry and relatively unproductive semi-natural grasslands, which are common in Sweden, are unlikely to sustain high earthworm diversity – a pattern contrasting to previously reported plant diversity responses. Instead, earthworm diversity seems concentrated to more productive grazed grasslands, with large within-grassland heterogeneity. Therefore, we highlight the importance of considering soil animals in conservation policies for semi-natural grasslands.

### 1. Introduction

In agriculturally managed land, Lumbricid earthworms

(Crassiditellata; Lumbricidae; Jamieson et al., 2002) contribute to important soil functions (Blouin et al., 2013). However, intensive agricultural management practices, such as conventional tillage, reduce

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earthworm density (individuals per unit area) and diversity (Briones and Schmidt, 2017). Reduced earthworm density and diversity may impair earthworm-mediated soil functions, such as nutrient mineralization (van Groenigen et al., 2019), carbon dynamics (Lubbers et al., 2017), and soil structure formation (Schon et al., 2017), as well as increase dependence on mineral fertilizer inputs (Chan, 2001). In agricultural landscapes, a lack of high-quality earthworm habitats may prevent the recovery of earthworm diversity in croplands, even if their management is adjusted towards earthworm friendly practices. This is because source populations are needed for earthworm recolonization (Lagerlöf et al., 2002). Field margins and herbaceous strips have been shown to be important for sustaining earthworm diversity in agricultural landscapes, although earthworm re-colonization of cultivated soils requires time and often drastically reduced management intensity (Crittenden et al., 2015; Frazão et al., 2017; Hof and Bright, 2010; Lagerlöf et al., 2002; Nuutinen et al., 2011; Prendergast-Miller et al., 2021; Roarty and Schmidt, 2013). Semi-natural grasslands, which have not been ploughed for decades or centuries, could also serve as earthworm diversity reservoirs. These habitats are sustained by agricultural practices like mowing or livestock grazing, and if abandoned from active use, they will eventually overgrow and revert to forest. Of Swedish semi-natural grasslands, more than 90% have been lost over the last century (Cousins et al., 2015). In themselves, semi-natural grasslands already have a high conservation value due to their importance for above-ground biodiversity (Dahlström et al., 2006; Dengler et al., 2014). However, earthworm diversity and density can vary widely between individual semi-natural grasslands (Rutgers et al., 2016). It is therefore essential to understand which grassland characteristics best support high earthworm diversity.

Grasslands harbour higher earthworm species numbers and densities than annually cropped arable land, as grasslands exhibit less soil disturbance, higher resource inputs, and more continuous shelter by the vegetation cover (Edwards and Arancon, 2022; Keith et al., 2012; Spurgeon et al., 2013). In pastures, the dung of grazing animals serves as a high-quality resource for earthworms and further supports their densities (Bacher et al., 2018). Semi-natural grasslands offer particularly promising refuge habitats for earthworms, because these habitats have a long continuity of no soil disturbance combined with mowing or grazing (Dengler et al., 2014). They also support diverse communities of plant species adapted to such conditions (Dahlström et al., 2006; Dengler et al., 2014), which may support higher earthworm diversity via variability in food resources (Spehn et al., 2000). Semi-natural grasslands also vary in soil moisture and nutrient conditions, tree and shrub density, and the type and level of management (i.e., the intensity of mowing or grazing; Söderström et al., 2001; Löfgren et al., 2020), which induces small-scale variation in earthworm microhabitats (Richard et al., 2012). On the other hand, a large part of the remaining semi-natural grasslands in Sweden are located on marginal lands, which were never suitable for, or have been abandoned from, crop production due to low productivity or difficulty to plough, e.g., due to many boulders and rocks (Eriksson and Cousins, 2014). Such grasslands typically sustain a high diversity of plant species tolerant to dry and nutrient-low soil conditions (Löfgren et al., 2020) but may be poor quality habitat for earthworms which are sensitive to desiccation (Edwards and Arancon, 2022).

Characterizations of earthworm communities in grasslands are mainly based on comparisons between grasslands and other habitat types (Boag et al., 1997; Decaëns et al., 2008; Didden, 2001; Keith et al., 2012), or between types of grassland management, such as fertilization regime (e.g. Timmerman et al., 2006; Curry et al., 2008; Singh et al., 2021). Far less is known about variation in earthworm communities among semi-natural grasslands linked to environmental conditions such as soil properties (but see van Vliet et al., 2007; Hoeffner et al., 2021), topography, and water conditions (but see Ivask et al., 2007; Hackenberger and Hackenberger, 2014), although this would be valuable knowledge for soil biodiversity conservation purposes. Indeed, there have recently been calls to better assess earthworms and other soil organisms in conservation policies (Guerra et al., 2021; 2022; Phillips

et al., 2019). Many Lumbricid earthworm species establish easily in new environments. This feature helped Lumbricid earthworms colonize Northern Europe after the last glacial period and contributes to their invasiveness via human introduction in many parts of the world (Edwards and Arancon, 2022). The high capacity of earthworms to establish in new areas makes them unlikely to be threatened on a global scale. Nonetheless, many Lumbricids may be threatened on national or regional scales due to their slow dispersal and sensitivity to intensive agriculture (Briones and Schmidt, 2017; Edwards and Arancon, 2022). However, too little is known about Lumbricid distributions, prevalence patterns, and habitat requirements at the species level, to verify these general claims.

One reason for the knowledge gaps in Lumbricid ecology is that morphological species determination depends on the presence of sexual organs and the clitellum (Richard et al., 2010). Therefore, earthworm juveniles, which lack these structures, are commonly excluded from analyses. This is a problem in ecological studies, since juveniles typically comprise the vast majority of individuals in the community (e.g. Emmerling, 2001; Marwitz et al., 2012). The juvenile fraction is also of particular relevance to community and population dynamics, as it is in this fraction that future regeneration and population replenishment resides. As an alternative solution, juveniles are sometimes classified to three ecological categories (epigeics, endogeics, anecics), defined by their presumed feeding and burrowing habits (Bouché, 1977; Bottinelli et al., 2020). However, these groups conceal substantial functional variation at the species level (Hoeffner et al., 2022), thus deflating the use of this classification for species conservation. Furthermore, molecular methods have revealed that several morphologically-described earthworm species comprise groups of cryptic species (Erséus et al., 2023; Martinsson and Erséus, 2017; Pérez-Losada et al., 2009). While these taxa look identical, they may have different ecologies and thus functional roles (Spurgeon et al., 2016). The exclusion of juveniles and the lack of resolution among cryptic species may therefore compromise insights from ecological studies. In particular, they will hamper estimation of diversity, distribution and prevalence patterns, and complicate any predictions regarding functional consequences of species loss. These issues can be overcome by using molecular methods such as DNA barcoding for species determination, but so far DNA barcoding has rarely been used in earthworm ecology (Decaëns et al., 2016; Decaëns, 2021; Huang et al., 2007).

To assess how specific environmental characteristics, including properties of soil, vegetation, management, and landscape, affect earthworm density, diversity and community composition, we compared earthworm communities in 28 northern temperate semi-natural grasslands in Sweden. To improve the resolution of the diversity estimates, we determined individuals in all age groups to species level through DNA barcoding. We specifically addressed the following questions:

- 1) Which of the selected environmental variables contribute to earthworm density and diversity?
- 2) Do earthworm species and ecological categories respond differently to variation in the selected environmental variables?
- 3) How large is the difference in resolution of earthworm species identification using DNA barcoding compared to morphological determination?

## 2. Material and methods

### 2.1. Study area

We sampled 28 semi-natural grasslands within 40 km from Uppsala (59° 52' N, 17° 39' E), south-central Sweden (Fig. 1). The semi-natural grasslands were typical of the area, with sparse trees and boulders and varying levels of grazing. Based on historical maps and aerial photographs, the selected grasslands were all at least 150 years old, with no

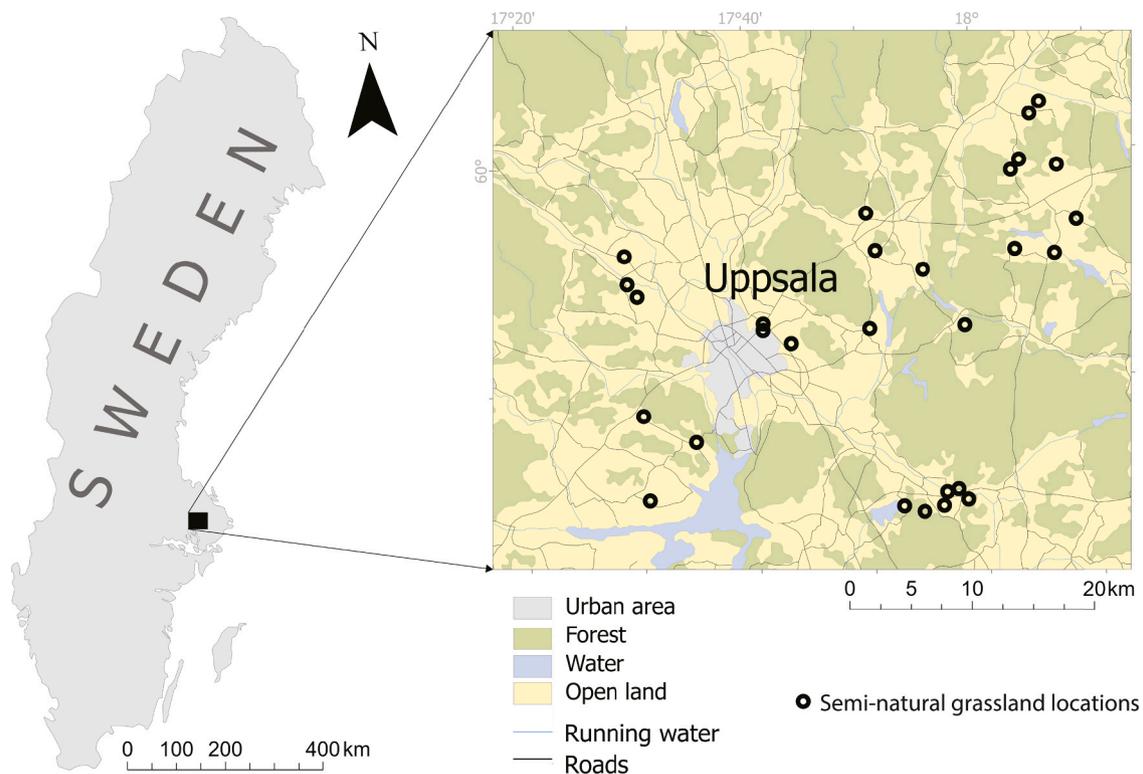


Fig. 1. The distribution of the 28 semi-natural grasslands in the area surrounding Uppsala city. The background map of Sweden is derived from ESRI global maps, whereas the land cover information in the inset map was adopted from the Land Survey General map of Sweden.

crop cultivation activities since at least the end of the 1800's. The grasslands were selected for previous studies on drivers of plant, insect, and bird diversity as a representative set from a larger number of grasslands in the region (Pärt and Söderström, 1999; Söderström et al., 2001; Vessby et al., 2001).

The climate of the study area is humid continental. During the past 30 years, the mean temperature in this region was 6.8 °C and the mean annual precipitation was 541 mm (Swedish University of Agricultural Sciences, Ultuna Weather Station, 1991–2020). During the earthworm sampling in early September, the mean daily temperature was 13.0 °C and the mean rainfall was 0.3 mm d<sup>-1</sup>.

The geological landscape in the study area is diverse (The Geological Survey of Sweden, Quarternary Deposits Map). The western part consists mainly of a plain dominated by fine sediments intersected by areas with moraine and bedrock outcrop, while the eastern part is more strongly dominated by moraine and bedrock outcrops intersected by areas with fine sediments or organic soils. Two major esker systems cut through the area in a north-south direction, along which sections of coarse sediments such as sand and gravel are distributed, especially in the western part. Most of the areas with fine sediments are used for agricultural production (The Land Survey, 1950's Economic Map).

## 2.2. Earthworm sampling and morphological identification

We sampled earthworms between 30th of August and 15th of September 2021, as soil moisture conditions typically favour earthworm activity in autumn (Edwards and Arancon, 2022). Within each grassland, we established a linear transect along which we took three samples 10 m apart (Fig S1). Based on aerial photos, we selected representative, flat areas that would not be too close to the grassland edge, shading trees, or shrubbery, or exposed to bedrock or extremely wet conditions. We took one earthworm sample at each location (i.e., 3 per grassland). For each sample, we dug up a soil block of 30 cm (width) × 30 cm (length) × 20 cm (depth) and immediately hand sorted the soil for

earthworms. To extract further earthworms from beneath each pit, we poured 2.5 l of allyl isothiocyanate (AITC) solution (100 mg AITC l<sup>-1</sup> water; Zaborski, 2003) onto the exposed pit bottom and collected emerging earthworms for 30 minutes. We rinsed the collected earthworm specimens in tap water and preserved them in 95% ethanol.

All preserved adult earthworms with a well-developed clitellum were morphologically identified to species level, using the key by Sherlock (2012). Juvenile individuals were identified to genus level. All individuals and severed parts were dried briefly with tissue paper, weighed, and placed individually in Eppendorf tubes filled with 95% ethanol. For severed parts of earthworms, we noted whether the part was a head or a tail, or from the middle of the individual. Later, we used this information to adjust the number of individuals per m<sup>2</sup> (see Section 2.3).

## 2.3. Earthworm identification with DNA barcoding

DNA was extracted and amplified following in-house protocols at Bioname Oy, Finland. The full procedure is described in Appendix 1. In short, DNA was extracted from a 3×3 mm piece of tissue from each sample using a mix of salt extraction buffer (0.4 M NaCl, 10 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 2% SDS; Aljanabi and Martinez, 1997; Vesterinen et al., 2016) and proteinase K (stock 20 mg ml<sup>-1</sup>; REF: 740506, Macherey-Nagel, Düren, Germany). The lysate mix was purified using magnetic SPRI-bead solution (Vesterinen et al., 2016). Part of the cytochrome oxidase subunit I (COI) gene was amplified using a primer mix with one forward and two reverse oligos (LCO1490, HCO2198, and COI-E; Bely and Wray, 2004; Folmer et al., 1994). For some samples, supplementary primers with a shorter target region were used due to unsuccessful amplification using the aforementioned primers. Details about these primers and the cycling conditions are described in Appendix 1. PCR products were purified using A'SAP clean kit (Arctic-Zymes, Tromsø, Norway), and Sanger sequencing was performed at Macrogen Europe B.V (Amsterdam, the Netherlands). For 41 (out of

2703) samples, sequencing was not successful even after trying several primers.

The forward and reverse sequence reads were assembled into contigs and quality assessed to achieve consensus sequences in Geneious 6.1.8 (Drummond et al., 2011). Successful sequences were identified using the reference sequences in the Barcoding of Life Database (BOLD) and GenBank (NCBI), where a similarity of 96% or higher was considered a match at species level (in most cases the matched sequences had a similarity of 98% or higher). The code names for the cryptic species follow Erseus et al. (2023). All successful sequences were uploaded to Genbank via BOLD.

Since many of the samples contained severed parts of earthworms, we adjusted the species densities per sampling location based on the species determinations by DNA barcoding. When both heads and tails of a species were collected in the same study location, we calculated the species density by subtracting the number of the less numerous of the two types of parts (heads or tails) from the total number of parts of that species in that study location. We excluded middle parts if there were heads and/or tails of the same species collected from the same study location. We calculated the number of undetermined individuals per study location by summing up the unsuccessfully determined samples per study location, excluding the parts that could have belonged to other individuals already counted from the same study location.

To characterize the relative representation of the three earthworm ecological categories defined by Bouché (1972), (1977) in each study location, we calculated community weighted means (CWM) for each sampling location and category. Earthworm species do not fall strictly within one ecological category only but can simultaneously have epigeic, endogeic and anecic characteristics. Thus, we used the proportions of ecological group characteristics for each species calculated by Bottinelli et al. (2020); Table 1). Specifically, we averaged the percentage characteristics of an ecological category across the species occurring in a study location, weighed by their density. As *Octocasion tyrraeum* was not included in the calculations by Bottinelli et al. (2020), we assigned it to the endogeic category based on other literature (Bisht et al., 2006; Jayasinghe and Parkinson, 2009), and assumed similar ecological category representation as for the two other endogeic species in our dataset, *Aporrectodea caliginosa* and *A. rosea* (Table 1). Cryptic species were assigned to the same ecological categories as the corresponding morphospecies.

## 2.4. Environmental parameters

To dissect environmental drivers of earthworm communities, we aimed to characterize four key aspects of the environment: chemical and physical properties of the soil, species composition and structure of the vegetation, grassland management, and landscape structure. Conceptually, we considered these four aspects to represent a hierarchy, where soil parameters represent the lowest and primary level, determining the immediate habitat of earthworms. Vegetation, grassland management, and the surrounding landscape then represent additional levels of variation. Soil, vegetation, and management can be assumed to influence earthworm communities via determining the living conditions and food resources for earthworms. The final level, landscape structure, shapes the regional species pool, and may affect local earthworm communities via edge effects and by adding variation to available resources. As will be evident below, both the environmental metrics compiled and the modelling approach adopted will reflect this conceptual framework. By selecting variables from each category in turn, rather than selecting variables from among the full set of variables at once, we avoided the problems of overfitting. We also deflated the high risk of finding spurious results when selecting variables from too many alternative models.

### 2.4.1. Soil properties

Earthworm communities are shaped by soil properties that define

their food resources and living conditions (Edwards and Arancon, 2022). Such soil properties include, e.g., organic matter content (SOM; van Vliet et al., 2007) and C:N ratio (de Wandeler et al., 2016), as well as pH (Johnston, 2019), bulk density (Capowiez et al., 2021), texture (Lapied et al., 2009; van de Logt et al., 2023), and water content (Singh et al., 2019). In addition, N and C isotopic ratios in soil organic matter likely reflect important determinants of earthworm resource availability and living conditions. Increased soil  $\delta^{15}\text{N}$  reflects ecosystem losses of inorganic N via denitrification or leaching, which is often correlated with increased N mineralization and inorganic N availability (Kahmen et al., 2008). Higher values of  $\delta^{13}\text{C}$  indicate drought stress and long-term low water availability in the soil, as plants fractionate less against the heavier isotope during  $\text{CO}_2$  assimilation during drought (Klaus et al., 2016). To determine these, we collected 15 soil samples within each grassland using a 3 cm cylindrical steel corer. We took the samples down to a depth of 20 cm or, in the presence of many stones, as deep as we could push the corer. We took five samples around each of the three earthworm sampling locations: one in the middle and four in cardinal direction at a distance of 1.5 m from the middle. All 15 cores were pooled per grassland.

We weighed and stored the pooled samples at  $-20^\circ\text{C}$  on the sampling day and later milled them frozen. A subsample was analysed for soil texture (contents of clay, silt and sand) and pH at Agrilab Uppsala, Sweden, following the Swedish standards SS-ISO 11 277 and SS-ISO 10 390, respectively. Another subsample (350 g) was freeze-dried at  $-20^\circ\text{C}$  for 72 h to obtain a homogeneous composite sample. We weighed the sample before and after freeze-drying, to calculate soil water content and bulk density ( $\text{g cm}^{-3}$ ). We ground the freeze-dried samples with a mortar and pestle, further sieved them with a 2 mm mesh to remove big stones or roots, and ball milled the subsamples for chemical analyses. Organic matter content was determined by loss-on-ignition at  $550^\circ\text{C}$  for 6 h, and total C and N content as well as 15 N:14 N and 13 C:12 C isotopic ratios ( $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ ) on an isotope ratio mass spectrometer (DeltaV, Thermo Fisher Scientific, Bremen, Germany) coupled to an Elemental analyzer (Flash EA 2000, Thermo Fisher).

### 2.4.2. Vegetation

Vegetation affects earthworm food resources and living conditions through the quantity, quality, and variability of organic material entering the soil, as well as the structure and chemical properties of the root system (Piotrowska et al., 2013; Spehn et al., 2000). Vegetation also determines the thickness and quality of the litter layer, which is the main habitat of epigeic earthworms (Patoine et al., 2020). Thus, we surveyed the vegetation to describe its quantity (vegetation height), quality (coverages of different functional groups), and variability (plant species richness).

Within each grassland, we surveyed the vegetation simultaneously with the earthworm sampling. The survey protocol is described in detail in Lundin et al. (2016). In short, we determined the occurrences of vascular plant species within a survey area of 3 m radius around each of the three sampling locations (Fig. S1). The coverages of legumes, other broad-leaved herbs, graminoids, graminoid litter, and mosses were visually estimated. We also described the vertical structure of the vegetation by estimating % cover of vegetation in three height classes:  $<5$  cm, 5–15 cm, and  $>15$  cm. These observations we converted to average vegetation height as weighted averages of the class mid-point values (3 cm, 10 cm and 25 cm). For estimation of plant species abundance, we determined a set of five small circular plots ( $0.25\text{ m}^2$ ) along a 5 m line at each sampling location perpendicular to the sampling transect (Fig S1). The abundance of each species was determined on a 1–6 scale, where the species occurring in the large, but not in any of the small circular plots, received abundance '1', and the species occurring in the small plots received the abundance of 1 + the number of small plots in which they occurred.

Since point measurements of soil properties may not be sufficient to characterize long-term fluctuations in soil moisture and nutrient

conditions, we also used the vegetation data to calculate Ellenberg indicator values for soil moisture, soil nutrients and light (Ellenberg, 1992). We calculated the indicator values for each sampling location as an average of the occurring species weighted by their abundance in the ordinal scale 0–6 (cf Diekmann, 2003).

#### 2.4.3. Grassland management

Grassland management, such as mowing and fertilization, indirectly affects earthworms, via its effects on vegetation and soil (Curry, 2004). Grazing animals, in turn, may affect soil structure, vegetation height, and litter layer thickness, via trampling and grazing, and earthworm food quantity and quality via dung production (Bacher et al., 2018; Hoeffner et al., 2021; Schlaghamerský et al., 2007).

We collected information about the management of the grasslands during the past five years before our sampling by direct contact with land managers. For each grassland, the fenced area, the numbers and species of grazing animals, and the timings of grazing periods per year were recorded. None of the grasslands was mowed or fertilized. Then, we calculated the average, relative grazing pressure per hectare per year for each grassland based on the estimated feed need of each grazing species in relation to dairy cows (“grazing animal unit”; Table S1) and the length of the grazing period per year. This estimate (average animal units  $\text{ha}^{-1} \text{yr}^{-1}$ ) was converted into a grazing index by applying an ordinal scale with seven levels 0–6 on the ranked estimates.

#### 2.4.4. Landscape

The surrounding landscape defines the regional earthworm species pool (Decaëns et al., 2008) and may affect earthworm communities via edge effects on resource and habitat variability (Hoeffner et al., 2021; Zeithaml et al., 2009). To describe landscape structure around the study locations, we determined two variables: habitat heterogeneity and grassland coverage within a defined radius around the sampling locations. We obtained the habitat data from the GIS-based landscape classification BIOTOP SE (Skånes, in preparation). This database combines data derived from sources such as colour infrared high-resolution aerial photos and the Quaternary deposits database from Swedish Geological Survey (SGU) with property and land use data from the maps and databases of the Swedish Board of Agriculture (Jordbruksverket), the Swedish Environmental Protection Agency (Naturvårdsverket) and the Land Survey (Lantmäteriet). For calculating habitat heterogeneity and grassland coverage, we aggregated the original biotope classes into broader categories, which characterize the most relevant landscape features, namely soil moisture regime and biotope type. The aggregated biotope classes are listed and described in Table S2.

We defined habitat heterogeneity as the Shannon diversity (Hill, 1973) of the aggregated biotope classes around the study locations. We calculated habitat heterogeneity for different radii with five m intervals 5–100 m from study locations in R (version 4.1.2; R Core Team, 2021) using the vegan package (Oksanen et al., 2020). Grassland coverage was determined as the proportion of semi-natural grassland (summed semi-natural grassland on different soil types, Table S2) within different radii from 5–100 m around the study locations. We selected the relatively short radii due to the limited mobility of earthworms (2.5–14 m  $\text{year}^{-1}$ ; Edwards and Arancon, 2022). For the models, we made the final selection of the radius for both habitat heterogeneity (20 m) and grassland coverage (25 m) by comparing the Akaike information criterion (AIC) of the multiple regression models of earthworm density (see Section 2.5.1) with landscape parameters calculated for different radii. We illustrate habitat heterogeneity within 40 m radius around the sampling locations with BIOTOP SE aggregated biotope classes and orthophotos in Fig. S2.

### 2.5. Statistical analyses

We used R (version 4.1.2; R Core Team, 2021) for all statistical analyses. To avoid the pitfalls associated with automated model selection

procedures (James and McCulloch, 2010) we selected the environmental variables for all models a priori. Using the rationale outlined above (see 2.4 Environmental parameters), we first hierarchically classified all measured variables into four groups, representing soil, vegetation, management and landscape. Means, standard deviations and ranges of all the measured variables are presented in Table S3. To avoid any flawed attribution due to collinear effects, we examined Pearson correlations between all environmental variables, averaged for each grassland (Fig. S3), and selected six uncorrelated variables as explanatory variables for downstream analyses. These included variables of each of the four variable groups: as indicators of soil conditions, we chose SOM, C:N ratio, and the Ellenberg indicator for soil moisture (hereafter referred to as the “moisture indicator”); as indicators of vegetation, management and landscape, respectively, we chose plant species richness, grazing intensity and habitat heterogeneity. Importantly, the choice of uncorrelated variables for analysis avoids the problems associated with attribution among confounded variables explaining the same variance. At the same time, this implies that we cannot separate the effects of the variables nominally included in the model from the effects of confounded (i.e. correlated) variables a priori excluded from the model. Thus, part of the effect of higher soil organic matter content may be due to higher water content or lower bulk density, and part of the effect of grazing intensity may be attributable to variation in vegetation height - a consideration to which we will return in the discussion section. Although pH did not significantly correlate with any of the above parameters, we excluded it from the models due to its small variation (5.2–6.4; Table S3), which was within the range of values regarded as suitable for most earthworm species (~4.5–7; Edwards and Arancon, 2022). All correlations among variables are presented in Figure S3. As an indicator of management, we had the choice to use grazing pressure either with or without transformation to an ordinal scale. To arrive at an objective decision, we fitted separate models using either metric and then selected the metric based on the ordinal scale, as yielding a lower Akaike information criterion (AIC) for the corresponding model.

To illustrate the impact of a particular variable on the focal response, we used two complementary measures of effect size: the variable-specific regression coefficient ( $\beta$ ), which indicates the change in the response with a unit change in the explanatory variable, and the standardized regression coefficient, which indicates the change in the response variable with a change of one standard deviation in the explanatory variable. For the latter, we regressed the response on variables normalized by subtracting their mean and dividing by the standard error. Since the latter response is scaled to the range of variation included in the material, it offers a measure more easily compared among drivers *sensu* “how large a change in the response is caused by typical variation in the driver”.

#### 2.5.1. Earthworm density, diversity and ecological categories

We used multiple linear regression models to examine how the selected environmental variables affect earthworm density and diversity in semi-natural grasslands. To define the response variables for the analyses, we calculated total earthworm density per square meter as well as extrapolated estimators for three complementary diversity indices (Hill numbers). The diversity indices include earthworm species richness (all species have the same weight regardless of their abundances), Shannon diversity (exponential of the Shannon index; the effective number of equally abundant species that would have the same diversity as the observed community) and Simpson diversity (the effective number of the dominant species) for each study location (Hsieh et al., 2016). The extrapolated diversity indices were calculated as Hill numbers using the iNEXT package (Chao et al., 2014; Hsieh et al., 2020) and modelled as a function of SOM, soil C:N ratio, moisture indicator, plant species richness, grazing intensity and habitat heterogeneity. All explanatory variables were treated as continuous, including the ordinal scale of grazing pressure. As our metric of habitat heterogeneity, we used

Shannon diversity of habitat types obtained for a radius of 20 m around the study locations, as based on a comparison of AIC values of earthworm density models where the only difference was the radius used for the landscape heterogeneity calculation. To account for multiple measures from the same grassland, grassland identity was included in the model as a random factor.

To test whether the representation of the three ecological groups of earthworms, epigeics, endogeics and anecics (Bouché, 1977, 1972) varies in response to the environmental variables, we constructed three regression models, one for each ecological category. As response variables in these models, we used the community weighted mean (CWM) of the three ecological categories in the sampling locations. To achieve a normal distribution of the residuals, the CMW of anecic earthworms was square root transformed.

All regression models were fitted using R package lme4 (Bates et al., 2015). We visually verified homoscedasticity and a normal distribution of the residuals.

### 2.5.2. Earthworm species niches

To characterize earthworm species niches, we fitted joint species distribution models to community data using the package Hmsc (Tikhonov et al., 2022). As response variable, we used the species  $\times$  site matrix of species records (presence/absence or abundance; see below) for each of the 84 sampling locations (i.e., 28 grasslands  $\times$  3 subsamples each). Species found in less than 10 earthworm individuals across all 84 samples (average density per sampling location  $< 1$  individual  $m^{-2}$ ; i.e., *A. caliginosa* L6, *A. trapezoides* 1, *Dendrobaena octaedra*, *L. festivus*, *L. rubellus* CEB, *L. rubellus* CEJ and *Octolasion tyrtaeum*) were excluded from the analyses resulting in the inclusion of 14 species in total. Due to the zero-inflated nature of the data, we applied a hurdle modelling approach, first fitting one model for presence-absence (PA) while assuming a probit distribution and then another model for abundance conditional of presence (ACOP) while assuming a Poisson distribution. In other words, we separately tested the contribution of selected environmental variables on species occurrences and their abundances in the sampling locations where they were present.

To adequately model impacts of different environmental features, we followed the hierarchical approach outlined above (see 2.4 Environmental parameters and 2.5.1 Earthworm density, diversity and community weighted means) for community-level descriptors. The same six variables whose effect on earthworm density and diversity we tested using multiple regression were now used as explanatory variables in the models (i.e. SOM, C:N ratio, moisture indicator, plant species richness, grazing intensity, and habitat heterogeneity). To sequentially account for effects of soil, vegetation, management, and landscape variables, we built four models for PA and four ACOP models ( $2 \times 4 = 8$  models). In the first hurdle model with each response type (PA or ACOP; models henceforth referred to as Model SoilPA and Model SoilACOP, respectively), we included soil properties only: SOM, C:N ratio, and the Ellenberg moisture indicator. In the second hurdle model (referred to as Model SoilVegPA and Model SoilVegACOP), we included the three soil variables from the previous step, now together with a descriptor of the vegetation, i.e., plant species richness. To the third hurdle model (Model SoilVegGrazPA and Model SoilVegGrazACOP), we further added grazing intensity as describing the effects of management on both vegetation and soil. To the fourth hurdle model (Model SoilVegGrazHetPA and Model SoilVegGrazHetACOP), we added habitat heterogeneity as a descriptor of the surrounding landscape. Since three samples were taken within each of the 28 grasslands, grassland identity was included as a random factor. The models were fitted with the default prior distributions (Ovaskainen and Abrego, 2020).

The posterior distribution was sampled with four Markov Chain Monte Carlo (MCMC) chains, which were run for 250 000 iterations, and thinned with 1000. The MCMC convergence was assessed examining the potential scale reduction factors (Gelman and Rubin, 1992) of the model parameters. We examined the explanatory and predictive powers of

each PA model through species-specific AUC (Pearce and Ferrier, 2000) and Tjur's  $R^2$  (Tjur, 2009) values. To compute predictive power, we performed 2-fold cross-validation at the grassland level, in which the samples were assigned randomly to two folds, and predictions for each fold were based on a model fitted to data on the other fold. To exclude unnecessary levels of complexity from the models, the final models were selected from the four alternatives for both types of models based on the highest average Tjur  $R^2$  (or AUC) for the PA models, and  $R^2$  for the abundance ACOP models.

## 3. Results

### 3.1. Earthworm densities and species numbers

In total, we collected 2419 earthworm individuals of which approximately 33% were adults and 67% were juveniles. We morphologically determined 29% of the individuals to the species level and 64% to the genus level. DNA barcoding allowed us to identify 99% of the individuals to the species level, including cryptic species, whereas 1% remained undetermined due to unsuccessful sequencing. The adult earthworms were morphologically assigned to 11 species (of which two, *Aporrectodea caliginosa* and *A. tuberculata*, are, in some literature, considered to form a single species; e.g. Sims and Gerard, 1985), whereas DNA barcoding revealed 21 species belonging to 13 morphospecies and encompassed both adults and juveniles (Table 1). The morphospecies revealed by DNA barcoding contained all species that were named by the morphological determination except *A. tuberculata*, which likely corresponds to one of the cryptic species in the *A. caliginosa* species complex (*A. caliginosa* L2). The two morphospecies that were not found in morphological determination were *Octolasion lacteum* and *O. tyrtaeum*.

Earthworm densities and the extrapolated diversity indices varied widely among the grasslands (Fig. S4–S7). Average earthworm density per grassland was 320 individuals  $m^{-2}$  (range: 96–692 individuals  $m^{-2}$ ). Earthworm species richness per grassland varied between 1.3 and 11.3 (mean 6.2) species, whereas Shannon diversity varied from a value of 1.2–7.8 (mean 4.2), and Simpson diversity from 1.1 to 6.3 (mean 3.3). The grasslands with the highest and lowest values were the same for all diversity indices.

**Table 1**

Earthworm species names and code names for associated cryptic species collected in this study. The code names for the cryptic species follow Erseus et al. (2023). For species with no cryptic species code, no separate cryptic species lineages have been detected in Scandinavia. The percentages of ecological category characteristics for each species are derived from Bottinelli et al. (2020), and reflect body pigmentation, size, coloration and ten other traits associated with the three categories. We assigned *Octolasion tyrtaeum* to be mainly endogeic based on Bisht et al. (2006) and Jayasinghe and Parkinson (2009), and estimated percentages of ecological category representation as averages of the other two mainly endogeic species, *A. caliginosa* and *A. rosea*.

Species	Cryptic species	% epigeic	% endogeic	% anecic
<i>Allolobophora chlorotica</i>	L2	30.9	37.7	31.4
<i>Aporrectodea caliginosa</i>	L2, L3, L6	15.9	79.8	4.3
<i>Aporrectodea longa</i>		32.0	0	68.0
<i>Aporrectodea rosea</i>	L1, L2, L4, RUE6.3, RUE6.4	14.7	85.3	0
<i>Aporrectodea trapezoides</i>	L1	49.3	0	50.7
<i>Dendrobaena octaedra</i>		97.4	2.6	0
<i>Lumbricus castaneus</i>		90.1	0	9.9
<i>Lumbricus festivus</i>		66.1	0	33.9
<i>Lumbricus rubellus</i>	CEA, CEB, CEJ	85.0	0	15.0
<i>Lumbricus terrestris</i>		30.1	0	69.9
<i>Octolasion cyaneum</i>		21.0	55.3	23.7
<i>Octolasion lacteum</i>	CEA	14.1	65.7	20.2
<i>Octolasion tyrtaeum</i>	CEA	15.3	82.6	2.2

### 3.2. Drivers of earthworm density and diversity

Earthworm density significantly decreased with an increase in the soil C:N ratio ( $\beta = -42.7 \pm \text{SE } 20.5$ ,  $t_{21.8} = -2.09$ ,  $P = 0.049$ ) and increased with an increase in the soil moisture indicator ( $\beta = 123.5 \pm \text{SE } 58.7$ ,  $t_{52.7} = 2.11$ ,  $P = 0.040$ ). Earthworm density was not significantly associated with soil organic matter content ( $\beta = -13.5 \pm \text{SE } 7.0$ ,  $t_{22.5} = -1.92$ ,  $P = 0.067$ ), plant species richness ( $\beta = -1.6 \pm \text{SE } 2.6$ ,  $t_{48.5} = -0.61$ ,  $P = 0.546$ ), grazing intensity ( $\beta = 9.0 \pm \text{SE } 13.5$ ,  $t_{19.6} = 0.67$ ,  $P = 0.513$ ) or habitat heterogeneity ( $\beta = 115.0 \pm \text{SE } 74.4$ ,  $t_{41.6} = 1.55$ ,  $P = 0.130$ ). When scaled to the amount of variation present in these drivers, we found that the moisture indicator had a larger relative effect size on earthworm density than had the C:N ratio (Fig. 2).

All earthworm diversity indices were positively associated with the soil moisture indicator (Species richness:  $\beta = 1.83 \pm \text{SE } 0.9$ ,  $t_{49.7} = 2.03$ ,  $P = 0.048$ ; Shannon diversity:  $\beta = 1.26 \pm \text{SE } 0.5$ ,  $t_{56.5} = 2.36$ ,  $P = 0.021$ ; Simpson diversity:  $\beta = -0.46 \pm \text{SE } 0.2$ ,  $t_{23.2} = -2.78$ ,  $P = 0.011$ ). Species richness also significantly increased with higher habitat heterogeneity ( $\beta = 3.11 \pm \text{SE } 1.1$ ,  $t_{40.8} = 2.75$ ,  $P = 0.009$ ), and was nearly significantly associated with a lower soil C:N ratio ( $\beta = -0.60 \pm \text{SE } 0.3$ ,  $t_{25.5} = -2.01$ ,  $P = 0.056$ ) and with higher grazing intensity ( $\beta = 0.41 \pm \text{SE } 0.2$ ,  $t_{23.3} = 2.05$ ,  $P = 0.051$ ). In addition to the soil moisture indicator, Shannon and Simpson diversity increased with a lower soil C:N ratio (Shannon diversity:  $\beta = -0.50 \pm \text{SE } 0.2$ ,  $t_{24.0} = -2.68$ ,  $P = 0.01$ ; Simpson diversity:  $\beta = -0.46 \pm \text{SE } 0.2$ ,  $t_{23.2} = -2.78$ ,  $P = 0.011$ ), and Shannon diversity also increased with higher habitat heterogeneity ( $\beta = 1.39 \pm \text{SE } 0.7$ ,  $t_{45.3} = 2.04$ ,  $P = 0.047$ ).

When scaled to the amount of variation present in these drivers, we found habitat heterogeneity to have a larger relative effect size on earthworm species richness than had the moisture indicator (Fig. 3A). In terms of earthworm diversity indices, habitat heterogeneity and the moisture indicator had larger relative effects on Shannon diversity than did the soil C:N ratio (Fig. 3B), and the moisture indicator had a larger

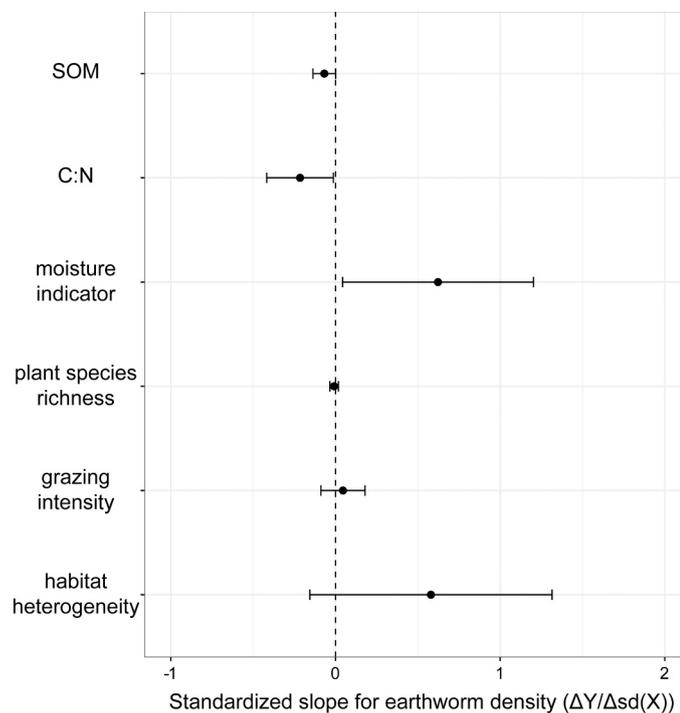


Fig. 2. Effect sizes (standardized slopes) for changes in earthworm density in response to changes in SOM, soil C:N ratio, moisture indicator, plant species richness, grazing intensity, and habitat heterogeneity. Standardized slopes and 95% confidence intervals are shown. The effect is significant where confidence intervals do not overlap with zero. The effect sizes were calculated normalizing the variables by subtracting the mean and dividing by the standard error.

relative effect on Simpson diversity than had the C:N ratio (Fig. 3C).

### 3.3. Drivers of ecological group representation

The representation of epigeic characteristics increased with an increase in soil organic matter content ( $\beta = 0.48 \pm \text{SE } 0.2$ ,  $t_{77.0} = 2.31$ ,  $P = 0.024$ ) and with an increase in the moisture indicator ( $\beta = 4.58 \pm \text{SE } 2.0$ ,  $t_{77.0} = 2.31$ ,  $P = 0.023$ ). The effect size of the moisture indicator was larger than that of SOM (Fig. 4). There was no significant association between any other environmental variables and epigeic representation; neither was anecic or endogeic representation significantly associated with any of the selected environmental variables (Fig. S8).

### 3.4. Earthworm species niches

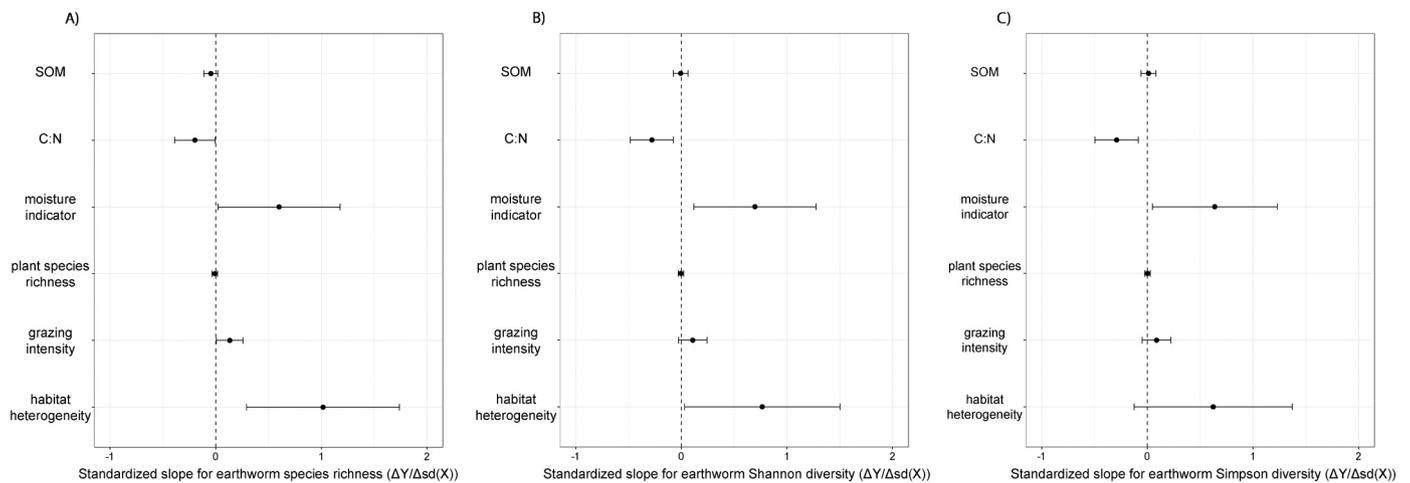
All eight joint species distribution models were successfully fitted to the data. Potential scale reduction factors were close to 1 in all models, which indicates good convergence (Table 2). Across all presence-absence models, three species stood out from the rest (*A. caliginosa* L2, *A. caliginosa* L3 and *A. longa*), as for them most variation in their occurrences was explained by grassland identity (Fig. 5A; Fig. S9). For the rest of the species, most variation was attributed to the soil moisture indicator and to the soil C:N ratio (Fig. S9). In abundance (conditional of presence) models, most variation was explained by soil organic matter content followed by the moisture indicator (Fig. 5B; Fig. S10).

Based on the highest Tjur  $R^2$  and AUC values (Table 2), we found model SoilVegGrazHetPA to provide the best descriptor of the drivers of species-specific presence-absence (Fig. 5A). This model included all six explanatory variables representing all four hierarchical variable groups, namely SOM, C:N ratio, moisture indicator, plant species richness, grazing intensity, and habitat heterogeneity. Based on the highest  $R^2$ , we found Model SoilVegGrazACOP to provide the best descriptor of species-specific abundance conditional on presence (Fig. 5B). This model included SOM, C:N ratio, moisture indicator, plant species richness and grazing intensity, but not habitat heterogeneity.

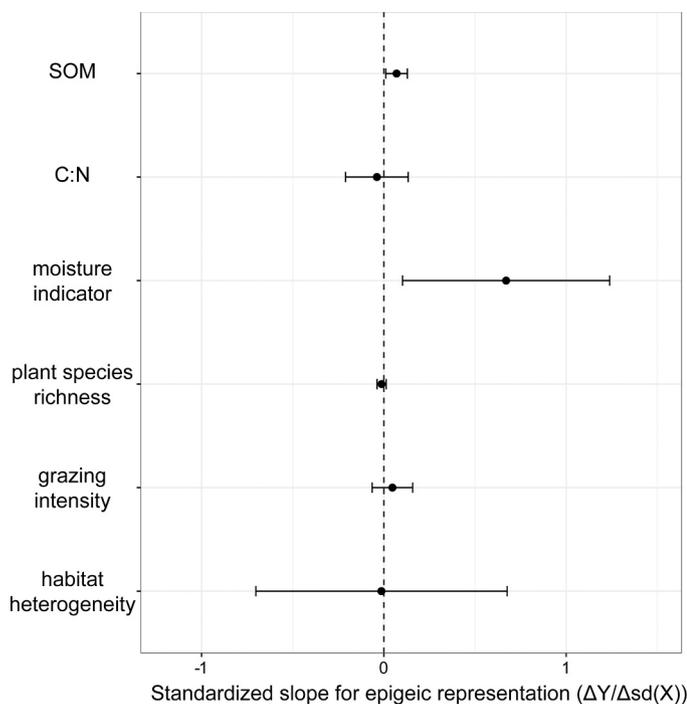
Five of the six environmental variables affected the occurrence of at least two different species with more than 95% posterior probability. For all significant impacts, the signs of species-specific responses were consistent across all species. For presence/absence, all species showing a detectable response were more likely to occur with a higher value of the Ellenberg moisture indicator, with higher grazing intensity and with higher habitat heterogeneity, and less likely with higher SOM and soil C:N ratio (Fig. 6A). For species showing a detectable response in terms of abundance (conditional of presence), abundances consistently decreased with higher SOM, and increased with a higher value of the Ellenberg moisture indicator (Fig. 6B).

## 4. Discussion

Lumbricid earthworms are some of the functionally most important components of the soil fauna. In this study, we introduce new resolution to identify the environmental determinants of earthworm abundance and diversity. By applying DNA barcoding, we were able to include the juvenile earthworms most frequently omitted from ecological analyses, and to distinguish between cryptic species. We found almost twice as many species when using DNA barcoding compared to morphological species determination, suggesting that earthworm species numbers have been largely underestimated. Drawing on the highly-resolved material, and on all age classes of earthworms, we find that environmental variables that indicate high soil fertility, such as high soil moisture indicator values and a low soil C:N ratio, predicted high earthworm density and diversity in semi-natural grasslands. Earthworm diversity also increased with higher small-scale habitat heterogeneity, thereby emphasizing the role of habitat diversity. Thus, grasslands characterized by these properties suit well for earthworm conservation and could serve as earthworm diversity reservoirs. In contrast, the dry and relatively



**Fig. 3.** Effect sizes (standardized slopes) for changes in earthworm A) species richness, B) Shannon diversity, and C) Simpson diversity in response to changes in SOM, soil C:N ratio, moisture indicator, plant species richness, grazing intensity, and habitat heterogeneity. Standardized slopes and 95% confidence intervals are shown. The effect is significant where confidence intervals do not overlap with zero. The effect sizes were calculated normalizing the variables by subtracting the mean and dividing by the standard error.



**Fig. 4.** Effect sizes (standardized slopes) for changes in community weighted mean (CWM) of epigeic characteristics in earthworm communities in response to changes in SOM, soil C:N ratio, moisture indicator, plant species richness, grazing intensity, and habitat heterogeneity. The mean effect sizes and the 95% confidence intervals for the respective variable are shown. The effect is significant where confidence intervals do not overlap with zero. The effect sizes were calculated normalizing the variables by subtracting the mean and dividing by the standard error.

unproductive areas typical of Swedish semi-natural grasslands suit poorly for earthworm conservation – despite their high value for the diversity of plant species (Eriksson and Cousins, 2014). Our results emphasize the importance of a multi-taxon approach in conservation of semi-natural grasslands, already pointed out by previous research (Söderström et al., 2001; Vessby et al., 2001), and the role of small-scale variation in earthworm habitat diversity. Below, we will discuss each finding in turn.

#### 4.1. DNA barcoding provides new resolution to earthworm ecology

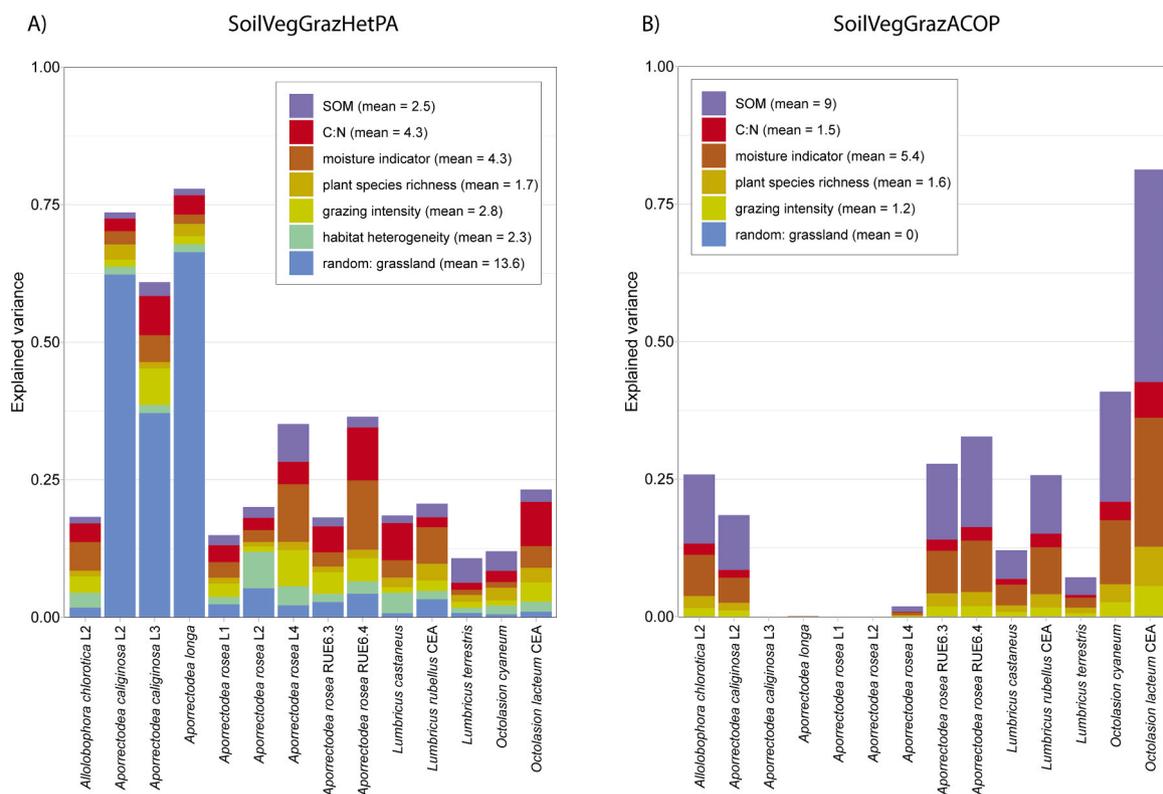
Previous research, using morphological species determination, has shown local earthworm species richness in Swedish and other temperate European grasslands to vary between about two and nine species in study areas of varying sizes (Decaëns et al., 2008; Frazão et al., 2017; Gormsen et al., 2004b; Lagerlöf et al., 2002; Nordström and Rundgren, 1973; Persson and Lohm, 1977; Richard et al., 2012; Schlaghamerský et al., 2007; Singh et al., 2021). Reliable comparisons of earthworm species richness between studies are a challenge due to a lack of standardized sampling methods and designs, variation in national and regional species pools, and the still unclear status of Lumbricid taxonomy (Decaëns, 2010; Edwards and Arancon, 2022). The average species richness of six per grassland, and especially the highest species richness of 11 species per grassland, found in our study, can be considered high. Nonetheless, there is no reason to believe that these grasslands would harbour higher earthworm diversity than those explored in previous studies. Indeed, the use of DNA barcoding revealed in total ten more species compared to morphological determination because it allowed us to distinguish between cryptic species (eight more species) and determine individuals that were mainly collected as juveniles or severely damaged during the sampling (two more species).

As is common in ecological studies of earthworms (e.g., Emmerling, 2001; Marwitz et al., 2012; Richard et al., 2010; Singh et al., 2021), a major part (67%) of the collected individuals were juveniles. All these individuals, i.e., the vast majority of the material, would have been excluded from the species level and diversity analyses if we had confined ourselves to morphological species determination. This, and the large difference in species numbers using morphological determination compared to DNA barcoding, implies that the use of morphological species determination typical of prior research strongly hampers accuracy of earthworm ecological studies. The patterns uncovered highlight the importance of increasing the use of DNA barcoding in earthworm determination to improve resolution for research concerning earthworm ecology and conservation. This would be important especially for studies about species richness, and for determining distribution patterns and habitat preferences for sparsely occurring species, which are easily missed using morphological species determination (Richard et al., 2010).

**Table 2**

Model fit statistics for the hurdle models (presence-absence, PA, and abundance conditional of presence, ACOP) applied. In the Soil models, we included the soil-related explanatory variables, SOM, C:N ratio and the Ellenberg moisture indicator. In the SoilVeg models, we included plant species richness in addition to the soil variables. The SoilVegGraz model included the aforementioned soil and vegetation variables and grazing intensity. The SoilVegGrazHet included all aforementioned variables and habitat heterogeneity. As a metric of MCMC convergence for the  $\beta$  parameters of the alternative models, we show the potential scale reduction factors (PSRF; PSRF values close to 1 indicate good convergence; Brooks and Gelman, 1998). As measures of the explanatory and predictive power of the alternative models, we show Tjur  $R^2$ , AUC and  $R^2$  values.

Model	PSRF			Explanatory power			Predictive power		
	Min	Median	Max	Tjur $R^2$	AUC	$R^2$	Tjur $R^2$	AUC	$R^2$
Presence-Absence (PA) models									
SoilPA	1.00	1.01	1.05	0.27	0.85		<0.01	0.50	
SoilVegPA	1.00	1.00	1.01	0.28	0.87		0.01	0.53	
SoilVegGrazPA	1.00	1.00	1.15	0.29	0.87		<0.01	0.53	
SoilVegGrazHetPA	1.00	1.00	1.10	0.30	0.87		0.05	0.56	
Abundance conditional of presence (ACOP) models									
SoilACOP	1.00	1.00	1.01			0.18			0.05
SoilVegACOP	1.00	1.02	1.37			0.18			-0.09
SoilVegGrazACOP	1.00	1.00	1.19			0.23			0.08
SoilVegGrazHetACOP	1.00	1.01	1.11			0.19			-0.02

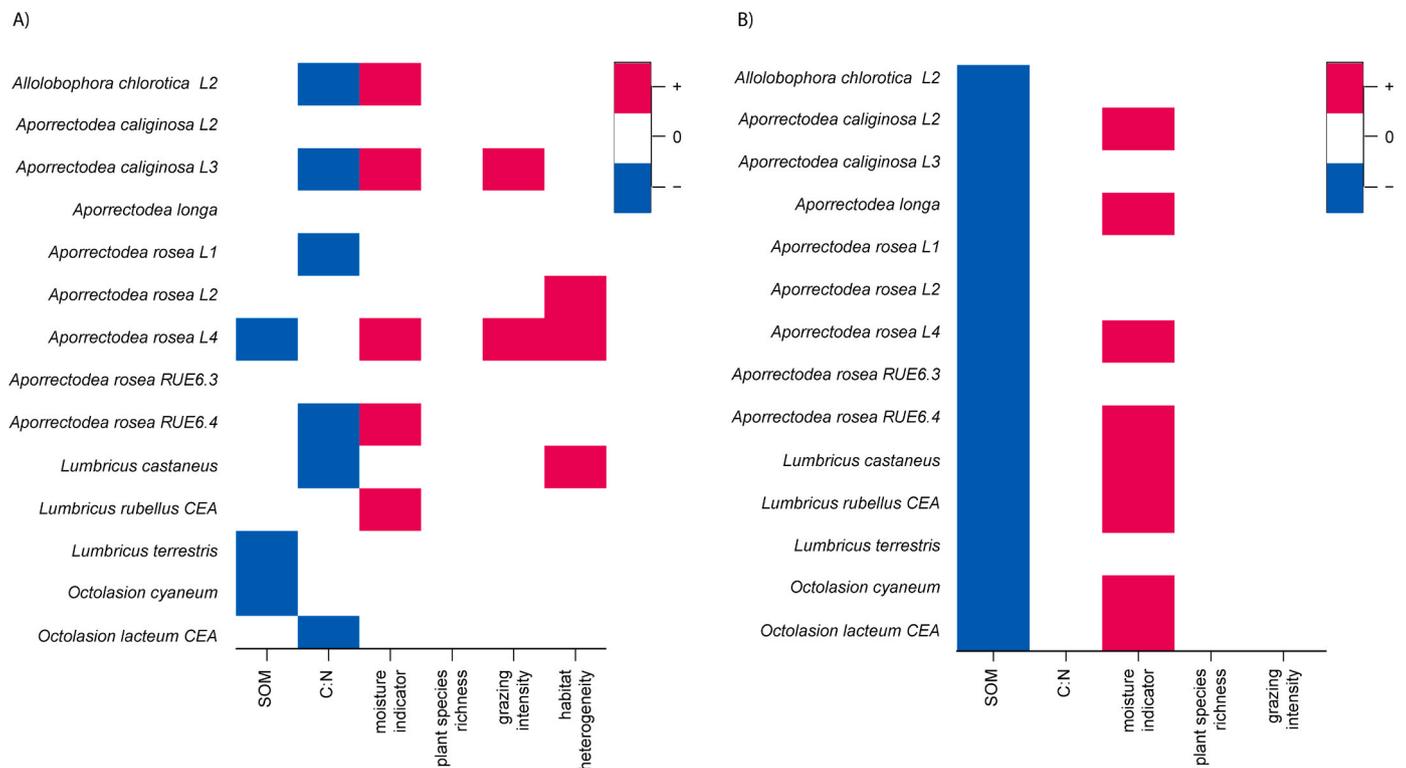


**Fig. 5.** Variance partitioning of the selected explanatory variables in A) the model SoilVegGrazHetPA, and B) the model SoilVegGrazACOP. The SoilVegGrazPA model is a presence-absence model including all the selected soil-related variables (SOM, C:N ratio and moisture indicator), plant species richness, grazing intensity, and habitat heterogeneity. The SoilVegGrazACOP is an abundance conditional of presence model including the soil-related variables, plant species richness and habitat heterogeneity. The bar height corresponds to explanatory power for each species (measured by Tjur  $R^2$  for the PA model and by  $R^2$  for the ACOP model). The identity and average variance proportion for each explanatory variable over all species is shown in the legends. Corresponding figures for all four PA models and all four ACOP models are presented in Fig. S9 and S10, respectively.

#### 4.2. Earthworm densities are favoured by high soil moisture and low soil C:N ratio

By including the full material derived above, we recorded a mean density of 320 individuals  $m^{-2}$ , ranging from 96 to 692 individuals  $m^{-2}$  across grasslands. The densities recorded are largely in line with previous studies, with the lowest densities matching those measured in cultivated soils in the same area (51–124 ind  $m^{-2}$ ; Torppa and Taylor, 2022), whereas the highest densities are more than five times greater. Indeed, earthworm densities in temperate grasslands vary widely, and can be as high as 4000 individuals  $m^{-2}$ . The highest densities are

reached in favourable conditions such as under cowpats (Bacher et al., 2018), whereas more typically, earthworm densities in temperate grasslands in Sweden and elsewhere in Europe vary between about 100 and 500 individuals  $m^{-2}$  (Decaens et al., 2008; Didden, 2001; Gormsen et al., 2004a; Hoefner et al., 2021; Ivask et al., 2007; Keith et al., 2018; Lagerlöf et al., 2002; Persson and Lohm, 1977; Spurgeon et al., 2013; van Vliet et al., 2007). There is some evidence that earthworm densities in semi-natural grasslands and permanent pastures can be higher, about 600–800 individuals  $m^{-2}$  (Rutgers et al., 2009). Since not all grasslands support equally high earthworm densities or diversity, it is important to characterize the drivers of density variation.



**Fig. 6.** Species responses to the selected environmental variables according to A) the PA model and B) the ACOP model. Positive responses with 95% posterior probability are shown in red, and negative responses with 95% posterior probability are shown in blue.

We found earthworm densities to increase with higher soil moisture, indicated by the Ellenberg indicator values of the plant community, and lower soil C:N ratio. It is well-established that soil moisture and factors affecting it, such as season (Singh et al., 2021), precipitation (Butt et al., 2022; da Silva et al., 2020) and soil texture (Rutgers et al., 2009; van de Logt et al., 2023), are important for earthworms. Although some species can escape drought or enter quiescence during dry periods, earthworm activity and reproduction relies on soil moisture, as earthworms breathe through their skin and are susceptible to desiccation (Edwards and Arancon, 2022). Indeed, Phillips et al. (2020) found climate to be one of the major drivers of earthworm diversity at the global scale. Ellenberg indicator values are a feasible way to characterize long-term soil conditions (Diekmann, 2003). Indeed, we did not find Ellenberg soil moisture indicator to correlate with soil moisture measurements during our sampling campaign, which represent a short-term moisture descriptor. Instead, the soil moisture indicator correlated negatively with  $\delta^{13}\text{C}$ , which indicates water deficiency stress in plants (Klaus et al., 2016), and positively with soil clay content, graminoid coverage and vegetation height. Thus, the strong association between the high moisture indicator and earthworm density likely reflects the importance of long-term water availability for earthworms. This is a promising finding with potential for application in earthworm monitoring and research, as it suggests that earthworm community properties, such as earthworm density can be predicted based on plant community structure.

Earthworm communities are often resource limited (Curry, 2004), and factors indicating high resource availability – such as soil organic matter content (Hoeffner et al., 2021; van Vliet et al., 2007) and, in agriculture, inputs of organic amendments (Briones and Schmidt, 2017) – often indicate high earthworm density. Similarly, soil properties indicating high productivity, such as low C:N ratio (de Wandeler et al., 2016) and neutral pH, have been shown important for earthworm density and community structure (Johnston, 2019; Singh et al., 2020). Our finding that earthworm density increased with decreasing soil C:N ratio is thus in line with these findings. Furthermore, we reiterate that our approach falls short of separating the impact of correlated variables.

Thus, part of the perceived impact of Ellenberg moisture indicator values may be due to the actual impact of variables positively correlated with it such as Ellenberg nutrient indicator values, vegetation height and graminoid coverage (Figure S3). These correlations will also signal the importance of ecosystem productivity for earthworms. We did not find an association between soil organic matter content and total earthworm density, although this relationship has been found in other studies (Hoeffner et al., 2021; van Vliet et al., 2007). Low variation in soil organic matter content is not a likely explanation for this, as the variation between grasslands was higher in our study (9.5–25.9%; Table S3) than e.g., in van Vliet et al. (2007); 5.0–10.2%. A possible reason for the lack of association between soil organic matter content and total earthworm density in our study is that SOM content was high enough in all grasslands, making other parameters more limiting. On the other hand, epigeic earthworms were relatively more abundant with increasing SOM content. This could be related to high SOM reflecting another unmeasured variable that could be detrimental to other groups of earthworms. For example, high vegetation density has been found to reduce anecic earthworms potentially due to hampering their surface foraging (Eisenhauer et al., 2009). Epigeic earthworms were also relatively more abundant when the soil moisture indicator was high, which is likely due to their litter dwelling lifestyle and inability to escape drought into deeper soil (Eggleton et al., 2009).

#### 4.3. Earthworm diversity is augmented by increased soil moisture, fertility and habitat heterogeneity

Interestingly, we found the same factors which promoted overall earthworm densities, namely the soil moisture indicator and soil C:N ratio, to also promote earthworm diversity. Specifically, earthworm species richness, Shannon and Simpson diversity all increased with increasing values of the soil moisture indicator, and Shannon and Simpson diversity also increased with decreasing soil C:N ratio, which indicates increasing soil fertility. In addition to the soil moisture indicator and soil C:N ratio, higher habitat heterogeneity within 20 m from

the sampling location also promoted higher earthworm species richness and Shannon diversity, and had a larger effect size on them than the two other variables.

Studies exploring drivers of earthworm diversity among grasslands remain rare, but the importance of soil moisture and C:N ratio agrees with global studies which have highlighted the importance of climate and pH (which is also associated with soil fertility) on earthworm diversity (Johnston, 2019; Phillips et al., 2020). The increase in earthworm species richness and Shannon diversity with habitat heterogeneity is also in accordance with previous research, which has shown habitat heterogeneity to be especially important for earthworm communities on small spatial scales (<20 m; Jiménez et al., 2012). Earthworm communities typically show aggregated distribution patterns, which has been attributed to both variation in soil microhabitats and species competition (Decaëns et al., 2008; Decaëns and Rossi, 2001; Richard et al., 2012). Hoeffner et al. (2021) found that presence of a hedgerow increased earthworm species richness in 1–2-year-old but not in older grasslands, suggesting that this type of increase in habitat heterogeneity can affect earthworm diversity especially soon after conversion of arable land to grassland. According to our study, variability in surrounding habitat types can benefit earthworm diversity also in old grasslands. On the other hand, an increase in shrub vegetation can be expected to increase earthworm diversity only until a certain threshold, after which the community structure likely switches to be dominated by a few forest-associated species such as *L. rubellus* (sensu lato) and other epigeic species (Pizl, 1992). Further research is necessary to clarify which elements in habitat heterogeneity increase earthworm diversity, and at what stage succession of a grassland to a forest may start to decrease earthworm diversity.

#### 4.4. Variation in community structure reflects responses by individual species

In addition to promoting earthworm density and diversity, the moisture indicator and soil C:N ratio were associated with earthworm species niches, explaining the largest part of variation in the occurrence of most earthworm species. The species with detectable responses to these variables were consistently more likely to occur in grasslands with higher soil moisture indicator values, lower soil C:N ratios, and higher habitat heterogeneity. These results are in line with our finding that these variables also promoted earthworm density and diversity. In addition, certain species were more likely to occur with higher grazing intensity (*A. caliginosa* L3 and *A. rosea* L4), and with lower soil organic matter content (*A. rosea* L4, *Lumbricus terrestris*, and *O. cyaneum*). For species abundance (conditional of presence), soil organic matter content and the moisture indicator explained most of the variation, with all species responding negatively to SOM and those with detectable response relating positively to the moisture indicator. Although the dung of grazing animals is a high quality resource for earthworms (Bacher et al., 2018), previous studies have found intensive grazing, via trampling and reduced litter layer, to be often detrimental to epigeic earthworms (Cluzeau et al., 1992; Schläghamerský et al., 2007; Schon et al., 2010) and sometimes also to species from other ecological groups (Cluzeau et al., 1992; Hoeffner et al., 2021). The species that we found to benefit from more intensive grazing were endogeic, and they may have benefitted from the dung of the grazing animals without suffering from the trampling and grazing effects. For soil organic matter content, negative association with *L. terrestris* density has been observed before (van Vliet et al., 2007). However, in contrast to our results concerning the endogeic *A. rosea* and *O. cyaneum*, endogeic earthworms have previously been shown to increase in density and species richness with increasing SOM (Hoeffner et al., 2021; van Vliet et al., 2007). As speculated earlier, higher soil organic matter content may also reflect higher vegetation density, which have been shown harmful for anecic earthworms (Eisenhauer et al., 2009) and, if also reflected in dense and deep-reaching roots, could also hamper endogeic earthworm activity.

The occurrences of three species, *A. caliginosa* L2, *A. caliginosa* L3 and *A. longa*, were best explained by the grassland identity. For *A. longa* this pattern is likely explained by mass occurrence in one of the grasslands due to accidentally sampling a degrading cowpat. For *A. caliginosa* L2 and *A. caliginosa* L3, which appear to occur in a non-overlapping manner at most grasslands, the pattern could be explained by competition between these cryptic species.

## 5. Conclusions

We found highly variable earthworm density and diversity in north temperate semi-natural grasslands, with record high values for Sweden in certain grasslands, and low values similar to intensively managed cultivated fields in others. In contrast to plants, earthworm diversity was highest in moist, fertile grasslands characterized by high small-scale variation in habitat types. Furthermore, the same environmental properties were associated with the occurrences of many of the detected earthworm species. Thus, semi-natural grasslands characterized by high soil moisture, fertility and habitat heterogeneity are likely to increase earthworm species diversity in agricultural landscapes, whereas dry, unproductive grasslands serve poorly for earthworm diversity conservation. Many, although not all, semi-natural grasslands may thus serve as earthworm diversity reservoirs. In addition, the remarkably higher number of earthworm species using DNA barcoding compared to morphological species determination demonstrates that research on earthworm diversity would benefit from adopting this more accurate method. This would be especially important in earthworm monitoring and species ecological research, to avoid confounding species with each other, and to acquire more realistic data on species diversity and distributions.

## CRedit authorship contribution statement

**Torppa Kaisa:** Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing, Data curation. **Glimskär Anders:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Writing – review & editing, Supervision, Writing – original draft. **Skånes Helle:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Visualization, Writing – review & editing, Supervision, Writing – original draft. **Klinth Márten:** Data curation, Methodology, Validation, Writing – review & editing, Formal analysis, Writing – original draft. **Roslin Tomas:** Methodology, Supervision, Writing – review & editing, Validation. **Castaño Carles:** Data curation, Investigation, Writing – review & editing, Methodology, Writing – original draft. **Taylor Astrid R.:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. **Viketoft Maria:** Supervision, Writing – review & editing, Resources, Validation. **Clemmensen Karina E.:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing, Formal analysis. **Maaroufi Nadia I.:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

## 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

The data that support the findings of this study and the accession numbers to the sequence data of the specimens are openly available in Zenodo at <https://doi.org/10.5281/zenodo.10360722> (Torppa et al. 2023).

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.agee.2023.108836](https://doi.org/10.1016/j.agee.2023.108836).

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