Environmental DNA is more effective than soil-pit hand sorting in evaluating earthworm biodiversity responses to more regenerative agricultural management

[1]Llanos, J., [2]Hipperson, H., [2]Horsburgh, G., [3]Lappage, M., [2]Maher, K. H., [1,2]Burke, T., [4]Leake, J. R., [1]Watt, P. J.

[1]Ecology and Evolutionary Biology, School of Biosciences, University of Sheffield, Sheffield, UK.

[2]NERC Environmental Omics Facility, Ecology and Evolutionary Biology, School of Biosciences, University of Sheffield, Sheffield, UK.

[3]Biological Sciences, University of Leeds, Leeds, UK

[4]Plants, Photosynthesis and Soil, School of Biosciences, University of Sheffield, Sheffield, UK.

Abstract

Restoring and maintaining soil biodiversity is important for the sustainability of our food systems, particularly as the health of agricultural soils continues to degrade at alarming rates. Earthworms are key components of soil biodiversity and provide many benefits to agricultural soil functioning, but effective and standardised approaches to monitor their populations are lacking. In this study, the feasibility of using soil environmental DNA (eDNA) metabarcoding for surveying earthworm populations in agroecosystems was explored. Soil samples were collected from arable fields with temporary grass-clover ley strips sown into them, to investigate the effects of different land use treatments on earthworm communities. Extracellular earthworm eDNA was extracted, amplified and sequenced using next-generation sequencing, and earthworm populations were also sampled using a traditional hand-sorting approach. This allowed the performance of the eDNA method to be assessed and quantitative comparisons to be made between the different methods. The eDNA method detected significantly higher local species richness than the traditional hand-sorting approach, with the site occupancy proportions indicating that eDNA was markedly better at detecting anecic (deep

burrowing) earthworms. The high sensitivity of eDNA sampling enabled the detection of differences in the earthworm communities resulting from different land-use treatments in the same field, with significantly higher earthworm diversity detected in the 33-month old ley strips than in the adjacent arable soils. Both relative abundances and site occupancy proportions were used as potential proxies for abundance, and the performance of each of these measures and the implications for further work are discussed. eDNA sampling has potential as a powerful tool for monitoring agricultural earthworm diversity and soil health, and could be utilised on farms to promote better management and soil care.

Introduction

Humanity derives 98.8% of itsr food from soils (Kopittke et al., 2019), and so protecting and enhancing this resource will be essential for feeding the predicted 9.7 billion people on the planet in 2050 (UN, 2019). Soils are being degraded across the globe, and this has been attributed to multiple factors, including intensive agriculture, climate change and land use change (Lal, 2013; Muñoz-Rojas et al., 2017; Zhao et al., 2005). Because of this, there is widespread concern for the ability of humankind to meet future food security challenges (Jie et al., 2002; Tamene et al., 2019). Borrelli et al. (2017) estimates that 35.9 Petagrams of soil are lost globally per year, with 50.5% of total soil erosion occurring in cropland in 2012, despite it only accounting for 11.2% of the land area studied. For England and Wales, the economic cost of soil degradation has been calculated at around £1.2 billion per year (Graves et al., 2015).

In light of the need to address soil degradation, in recent years there has been an increasing focus on developing tools and indicators to accurately measure soil health and quality (Karlen et al., 2019; Liu et al., 2020). Recent policy developments are also looking to promote the establishment and uptake of comprehensive soil health monitoring in agroecosystems, including England's Environmental Improvement Plan and the upcoming EU Soil Health Law (DEFRA, 2023; European Commission, 2022). A number of useful tools and methodologies have already been developed to measure factors that contribute to soil health, including physical measures such as bulk density and macroporosity, and chemical measures such as pH and nutrient status (for a discussion of various methodologies, see Rinot et al., 2019). Biological factors also make up a crucial element of soil health, and measurements such as microbial activity and diversity have been proposed as potential biological indicators that

could be incorporated into monitoring schemes. Microbes are not the only organisms that are critical to determining soil health, however, and macrofauna such as earthworms also play an important role in many temperate soils.

Earthworms are recognised as 'ecosystem engineers' for the disproportionate impact they have on the soil environment and pedogenesis (Cunha et al., 2016; Lavelle et al., 1997). As a result of their burrowing and casting, earthworms create macropores and enhance the formation and restructuring of macroaggregates, which can increase soil water holding capacity and gas exchange (Blanchart et al., 1999; Hallam and Hodson, 2020; Schaik et al., 2014; Sheehy et al., 2019; Zhang and Schrader, 1993). Via their feeding, earthworms are responsible for the breakdown of organic matter and the distribution of it throughout the soil profile, which at the same time increases the availability of other nutrients that are important for the development of plants and microbes (Chaoui et al., 2003; Lavelle et al., 1998; Le Bayon and Milleret, 2009). The biological properties of soils are also greatly affected by earthworms, as a result of their direct or indirect interactions with other organisms and their ability to stimulate microbial activity (Bart et al., 2019; Binet et al., 1998; Monroy et al., 2011; Wang et al., 2020). Given the many ways that earthworms affect the dynamics of temperate soils, it is no surprise that they also bring great benefits to agriculture too (Bertrand et al., 2015). A meta-analysis by van Groenigen et al. (2014) found that as well as maintaining and promoting agricultural soil health, the presence of earthworms increased crop yields by an average of 25%, with higher increases seen in nitrogen deficient soils or those that had previously been disturbed and lost their structure.

Despite the immense contribution of earthworms to soil health in agroecosystems, the development of tools to monitor their populations has lagged behind that of other soil health indicators, and their populations remain understudied and largely unmapped. Efforts have been made to rectify this both at international and local levels, including research that has mapped global distribution patterns of earthworm biodiversity (Phillips et al., 2019) and the more local #60minworms UK study that tasked farmers with sampling and recording earthworm numbers in their fields (Stroud, 2019). However, progress towards increasing earthworm diversity sampling and population monitoring may be hampered by the limitations of traditional earthworm sampling techniques, which can be time and energy intensive, difficult to standardise, prone to biases and require taxonomic expertise (Andriuzzi et al., 2017; Bartlett et al., 2010; Čoja et al., 2008; Jiménez et al., 2006). Addressing these limitations and developing more standardised methods for sampling earthworm populations is therefore seen as important for monitoring their distributions and realising their potential, as both indicators and promoters of soil health.

Environmental DNA (eDNA) sampling is one possible alternative method for sampling earthworm populations that could address some of the limitations of traditional methods. eDNA sampling involves collecting, amplifying and sequencing genetic material that has been left behind by organisms in their environment, and which may originate from deposits such as hair, skin cells, mucus or faeces. The majority of studies employing eDNA sampling have tended to focus on aquatic environments (Belle et al., 2019), but the feasibility of applying eDNA sampling techniques to terrestrial environments has also been demonstrated, including through the sampling of ice cores, sediments, plant material, scats and air (for example Bohmann et al., 2011; Bohmann and Lynggaard, 2022; Hofreiter et al., 2003; Thomsen and Sigsgaard, 2019; Willerslev et al., 2007).

Soil is a potential reservoir of eDNA that can be sampled in order to build a picture of both above- and below-ground biodiversity. For example, Yoccoz et al. (2012) showed that soil eDNA profiles were consistent with above-ground plant diversity, and Andersen et al. (2012) sampled soil with known species compositions to demonstrate the viability of soil eDNA for vertebrate biodiversity sampling. Furthermore, Bienert et al. (2012) also demonstrated that eDNA can be used for sampling earthworm communities in undisturbed woodland and meadow soils. This research was later built on by Pansu et al. (2015), who showed differences in earthworm diversity at the landscape level in the Northern French Alps. However, to our knowledge earthworm eDNA sampling has yet to be applied to active agricultural soils, where it has the potential to be used for closer monitoring of earthworm populations and associated soil health. In fact, despite their importance for soil health, both soil meso- and macrofauna are seriously underrepresented in agricultural eDNA monitoring studies published to date, with just 7% of them describing work in this area and the vast majority (93%) focused on soil microbiota (Kestel et. al, 2022).

In this study, we aim to assess the performance of earthworm eDNA sampling in active agricultural systems as a potential tool to measure the diversity of in-field earthworm populations and to assess the effectiveness of soil health improvement interventions (temporary grass-clover leys). To do this, we collected soil samples and performed traditional earthworm hand-sorting in arable fields that had grass-clover ley strips sown into them. To sample earthworm eDNA from the soil, we used next-generation sequencing to obtain earthworm sequence numbers and species diversity, and compared these with the results from the traditional hand-sorting method and across different land use treatments. We explored the diversity patterns generated by the different methodologies, assessed the required sampling intensity and investigated some potential proxy measures of abundance. Finally, we discuss the potential effectiveness of earthworm eDNA sampling in agricultural systems for assessing soil health and monitoring the progress of soil management interventions.

Methods

Study site

Traditional earthworm hand-sorting and soil sampling for eDNA was performed at the University of Leeds Field Research Unit, a commercial mixed farm in West Yorkshire, United Kingdom (53° 52' 25.2 N 1° 19' 47.0" W). Sampling was done at the end of March/early April 2018 when the weather conditions were broadly similar (overcast, sunny spells, 7-12°C). Four arable fields were sampled, with each field containing two 70 m x 3 m strips that had been temporarily converted to ley (Figure 1). These ley strips were planted as part of the SoilBioHedge project to examine the impact of ley conversion and hedgerows on arable soil quality, and had been established for around 33 months at the time of our sampling. The ley strips were seeded with grass and clover seed mixtures, whilst the surrounding arable fields were in a conventional arable rotation dominated by wheat.

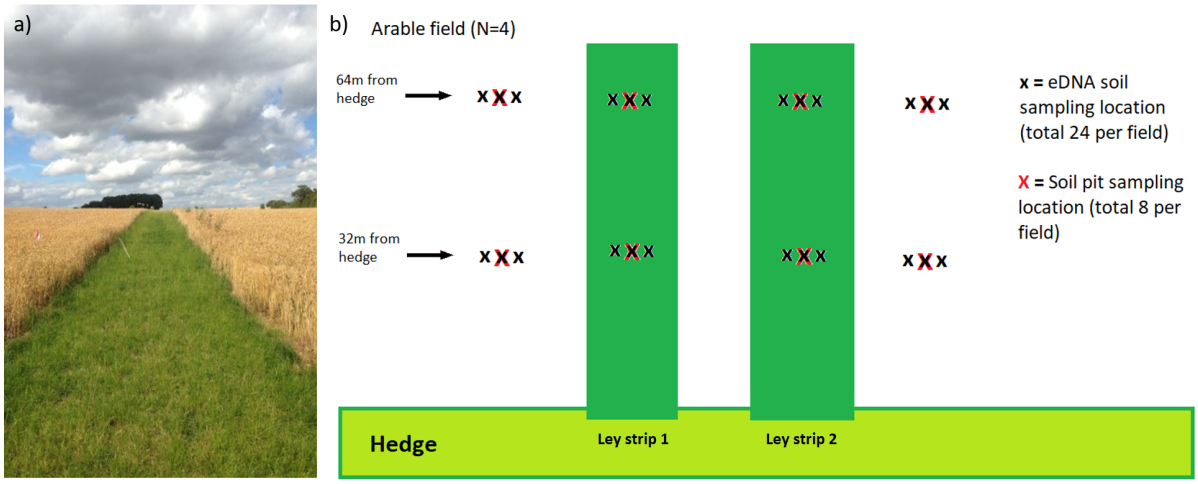


Figure 1. The study site and sampling protocol. a) one of the ley strips in an arable wheat field, b) the positions and numbers of samples taken per field, for both hand-sorting and eDNA sampling. This equated to a total of 32 pit samples for hand-sorting and 96 soil samples for eDNA analysis.

Traditional earthworm hand-sorting

Pit excavation for earthworm hand-sorting and eDNA soil sampling was done concurrently, with a total of eight soil pits excavated per field (24 pits in total, Figure 1). Four pits were dug in the ley strips and four in the arable field surrounding them, at distances of 32 and 64 m from the field margin.

Earthworm pits were dug by excavating an 18 x 18 x 15 cm block of soil and immediately placing it into a bucket with a sealable lid. After all earthworm pits and soil samples for eDNA analysis had been taken from a given field, the sealed buckets were transferred to a sorting site to be processed. The soil blocks were hand-sorted and all collected earthworms categorised into adults or juveniles before being preserved in 90% ethanol. Preserved earthworms were kept refrigerated at 4°C prior to species identification. The numbers of preserved adults and juveniles per pit were counted and individual earthworm weights recorded. Adults were then identified to species level under a dissecting microscope using morphological characteristics (Sherlock, 2018).

Soil environmental DNA sampling

Alongside the pit excavation (and prior to the handling of earthworms) smaller soil cores were extracted for soil eDNA analysis. These cores measured approximately 5 x 5 x 15 cm and were excavated using a pallet knife and a small trowel. Two were taken from 2 m either side of the larger soil pit and one was extracted from its centre, resulting in three soil samples for eDNA analysis per one traditional earthworm sampling pit (totalling 24 eDNA soil samples per field, Figure 1). The soil cores were given unique coded labels that identified which earthworm sampling pits they were associated with, so that the eDNA and traditional hand-sorting results could be directly compared. Each eDNA soil core was transferred to a transparent, sealable plastic sample bag and carefully broken up within it so that any stones, large pieces of plant matter, earthworms or cocoons could be removed. This process resulted in an average of ~175 g of soil per eDNA sample. To prevent possible contamination, between each soil core extraction individual tools were cleaned and soaked in 10% bleach for at least 15 minutes before being used again. Disposable gloves were worn and changed between every core extraction. At the end of each day, all equipment was cleaned again and hand tools were soaked in 10% bleach overnight. Excavated eDNA soil samples were stored at -20°C prior to eDNA extraction.

Earthworm environmental DNA extraction

eDNA extractions were performed using a modified protocol adapted from Taberlet et al. (2012, 2018). Soil samples were defrosted and thoroughly homogenised whilst still in sealed sample bags, by massaging the outside by hand. Fifteen grams of the homogenised soil was transferred to a 50 ml falcon tube. This step was repeated for each soil sample, so that two extraction replicates were taken for every soil sample. Freshly prepared saturated phosphate buffer (Na_2HPO_4, pH 8) was added to the

falcon tubes containing soil in a 1:1 ratio (i.e. 15 ml of buffer added) using a bulb pipette. For every three soil samples (totalling six extraction replicates), a negative extraction control, which consisted of phosphate buffer only, was included. The falcon tubes were rotated for 15 minutes, then centrifuged for 5 minutes at 4,700 rpm. Four hundred micolitres of the resulting supernatant was pipetted off and mixed with buffer solution from the Macherey-Nagel Nucleospin Soil kits. Reagents and filter columns from the Nucleospin Soil kits were used to complete the remaining extraction steps on a vacuum manifold. The resulting DNA extracts were diluted to 1:5 levels with low TE buffer and stored in sealed tubes at -20°C prior to amplification. Extractions were all performed in a pre-PCR room inside laminar flow cabinets, which were thoroughly cleaned before and after use with 10% bleach and exposed overnight to UV-C light. Disposable gloves, weighing boats and utensils were changed between the handling of each sample and all equipment was wiped clean with 10% bleach and exposed to UV-C light overnight.

Environmental DNA amplification, purification and sequencing

Two primer pairs were selected for use in this study, using primers described in Bienert et al. (2012) that amplify short sequences of mitochondrial 16S rDNA. The two primer pairs consisted of primers ewD and ewE and primers ewB and ewE. *In-silico* analysis of the primers was performed prior to selection, to ensure suitability.

For the initial amplification stage (PCR1), 2 µl of the diluted DNA extracts were mixed with 1 µl each of forward and reverse primers, 10 µl of Qiagen Multiplex PCR Master Mix and 6 µl of molecular biology grade sterile water, leaving a reaction volume of 20 µl. All forward and reverse primers were tailed with Illumina sequencing primers. The PCR1 mixtures were prepared inside laminar flow cabinets in a pre-PCR room and the reactions performed in individual 0.2 ml PCR tubes with sealable lids. In addition to extraction negatives, two PCR negatives containing ultrapure water instead of extracted DNA were included for every batch of reactions. For each batch, 20% of the extracted DNA samples were replicated (i.e. two PCR replicates produced for 20% of the samples) to enable reliability and sensitivity checks. After PCR1 the PCR product was run on a 1% agarose gel and visualised to ensure amplification had occurred, and to check for any contamination. PCR extracts were purified using AMPure XP beads. A second PCR step was then performed to add Illumina adapters and indexes and product sizes were checked using a TapeStation. The concentration of amplicons in each sample was then quantified using a fluorometer and the X number of samples were pooled together in equimolar amounts and a further bead clean undertaken. Each pool was quantified using qPCR and combined, resulting in one library for each primer pair. The BluePippin system was used to size-select

the final libraries to remove unwanted products and then the libraries were combined into a single final library for sequencing on an Illumina MiSeq.

Bioinformatics and statistical analyses

Sequencing quality was assessed using FastQC and MultiQC (Andrews, 2010; Ewels et al., 2016). Sequences underwent trimming using Trimmomatic (Bolger et al., 2014) to remove the Illumina adapter sequences from the data and trim lower-quality sequences. Reads were trimmed when the average Phred score dropped below 30 over a 4-base sliding window and reads below a minimum length threshold of 50 bp were discarded. MultiQC plots were then generated for the trimmed sequences to check that only high-quality data remained. The paired reads were aligned and converted to FASTA files using the FLASH alignment tool (Magoč and Salzberg, 2011), with maximum overlap and proportion of mismatches set to 150 bp and 0.1, respectively. The primer sequences were trimmed from the remaining sequences using the 'trim.seqs' command in the mothur software (Schloss et al., 2009), which was also used to label the sequences with their corresponding primer pair groups. After demultiplexing the sequences and producing separate FASTA files for each primer pair, USEARCH v9.2 (Edgar, 2010) was used to dereplicate the sequences, remove chimeric sequences and cluster highly similar sequences with 97% identity or greater. Unique mOTUs were generated for each primer pair and blasted against the NCBI nucleotide database, using a quality filter to only take forward hits with a maximum e-value of 0.00001 and 95% percentage identity (for the ewD/ewE primer pair this was increased to 97% percentage identity). The results were visualised using MEGAN 6 (Huson et al., 2016) and tables containing sequence ID and assigned taxon name were produced. Presence/absence and sequence number by sample matrices were then produced for each primer pair.

All statistical analyses were performed in R Studio using R version 4.0.1 (R Core Team, 2020). To investigate the effect of the different sampling methodologies and agricultural treatments on earthworm species richness, a linear model was fitted using the function 'lm' with sampling method, agricultural treatment and field ID as predictor variables and species richness as the response variable. Model assumptions were checked and plotted using the 'autoplot' function in the ggplot2 graphics package (Wickham, 2011) and post-hoc Tukey Honest Significant Difference tests carried out using the 'TukeyHSD' function. Bar charts were plotted using ggplot2 (Wickham, 2011). To further examine possible community composition differences across the different agricultural treatments and fields (and whether or not sampling methodology affected the picture observed), non-metric multidimensional scaling (NMDS) was performed on the relative abundances from each sampling

method using the 'metaMDS' function in the vegan package (Oksanen et al., 2013). The Bray–Curtis dissimilarity index was applied, and the ordinations were plotted with the environmental data overlaid using the 'ordihull' and 'orditorp' vegan functions. Shephard plots and stress-by-dimensions plots were also produced to check the suitability of the ordinations. One outlier site was removed from the ewB/ewE analysis as it skewed the NMDS2 axis and was deemed unrepresentative, likely as a result of low sequence numbers. PERMANOVA tests were used to calculate the statistical significance of any potential differences in community dissimilarity brought about by the treatment groups, using the function 'adonis2' in vegan. To evaluate the sampling intensities of each method and to check that the diversity present had been appropriately captured, species accumulation curves were plotted for each method, with one curve for each treatment type, using the 'specaccum' function in vegan. To investigate how closely the relative abundances of species in a sample compared across the different methodologies, scores per sample were plotted in stacked bar charts, overlayed with sequence numbers and abundances from traditional hand-sorting. Site occupancy proportions for each method were also calculated as potential proxies for overall species abundances.

Results

Traditional hand-sorting of earthworms from pits

A total of 718 earthworms was collected using the traditional hand-sorting method, 594 of which were juveniles (82.8% of the total numbers) and 124 were adults (17.2%). Morphological ID of the adults enabled 119 earthworms to be identified down to species level. Five earthworms could not be confidently identified due to damage inflicted during the sampling process. In total eight species were identified, with *Allolobophora chlorotica* the most common (65 individuals), followed by *Aporrectodea rosea* (15) and *Lumbricus castaneus* (11) (Table 1). Of the 119 adult earthworms morphologically identified to species level, 88 were found in the ley strips compared to 31 from the arable field samples.

Environmental DNA sampling

MiSeq sequencing yielded a total of 9,464,039 paired reads from the eDNA samples (including sequences from both primer pairs but not negatives and repeats). After the initial trimming based on sequence length and quality, 4,693,446 paired reads remained. On average 24.9% of reads were

removed during quality control. The sequences then underwent further quality filtering and selection, including the removal of chimeras and applying stringent BLAST criteria, which reduced the sequence numbers further.

After all bioinformatics clean-up and filtering steps had been completed, the total number of sequences classified to species level was 794,350 for ewB/ewE and 707,750 for ewD/ewE (not including the sequences obtained from negatives and repeats). For ewB/ewE, 464,287 were earthworm sequences and 330,063 were from the closely related *Enchytraeidae*, compared with 542,974 earthworm and 164,776 enchytraeid sequences for ewD/ewE. Theh eDNA primer pairs each detected eight earthworm species, with ewB/ewE identifying an additional nine enchytraeid species compared with eight for ewD/ewE. Of the eight earthworm species identified by both methods, the species with the highest sequence numbers overall was *A. chlorotica* (with 303,375 and 292,637 sequences found by ewB/ewE and ewD/ewE, respectively), followed by *A. rosea* (57,521 and 96,958) and *Aporrectodea longa* (35,524 and 50,800) (Table 1).

Sampling methods, agricultural treatment and earthworm diversity

Both eDNA primer sets and the traditional hand-sorting method identified eight earthworm species overall, with no single method picking up species that were not also found by the others. However, the mean earthworm species richness per pit sample was significantly affected by the sampling method (ANOVA: $F = 12.79$, $df = 2, 87$, $p < 0.001$), with traditional hand-sorting yielding lower earthworm species richness than both the ewB/ewE (Tukey multiple comparison test, $p = 0.006$) and ewD/ewE ($p < 0.001$) eDNA methods (Figure 2). There was no significant difference in mean earthworm species richness between the two eDNA methods ($p = 0.17$). Across all sampling methods, the land use treatment was also found to significantly affect species richness (ANOVA: $F = 57.78$, $df = 1, 87$, $p < 0.001$), with higher mean species richness per pit seen in the ley treatments compared with the arable fields (Tukey multiple comparison test, for hand-sorting $p = 0.008$, for both eDNA methods $p < 0.001$; Figure 2). Location-specific effects were also observed, as field ID was found to affect earthworm species richness (ANOVA: $F = 5.65$, $df = 3, 87$, $p = 0.001$), which was driven by significantly higher mean species richness in one field compared to another (Tukey multiple comparison test, $p < 0.001$). There were no further significant differences in mean earthworm species richness among the remaining fields.

Table 1. The total numbers of individuals and sequences found for each identified earthworm species, and the proportion of pit samples in which they were present (i.e. site occupancy), using the different eDNA or HS methodologies (HS = Hand Sorting).

Species	**No. individuals (From HS)**	**Total no. sequences (ewB/ewE)**	**Total no. sequences (ewD/ewE)**	**% of pits present (HS)**	**% of pits present (ewB/ewE)**	**% of pits present (ewD/ewE)**
Allolobophora chlorotica	65	303,375	292,637	74	97	94
Aporrectodea rosea	15	57,521	96,958	34	38	50
Lumbricus castaneus	11	25,415	37,120	28	31	38
Aporrectodea caliginosa	9	14,789	28,665	9	22	19
Satchellius mammalis	9	7610	10,228	19	6	19
Aporrectodea longa	7	35,524	50,800	19	47	44
Lumbricus terrestris	2	11,962	16,423	6	38	53
Octolasion cyaneum	1	8090	10,137	3	6	13

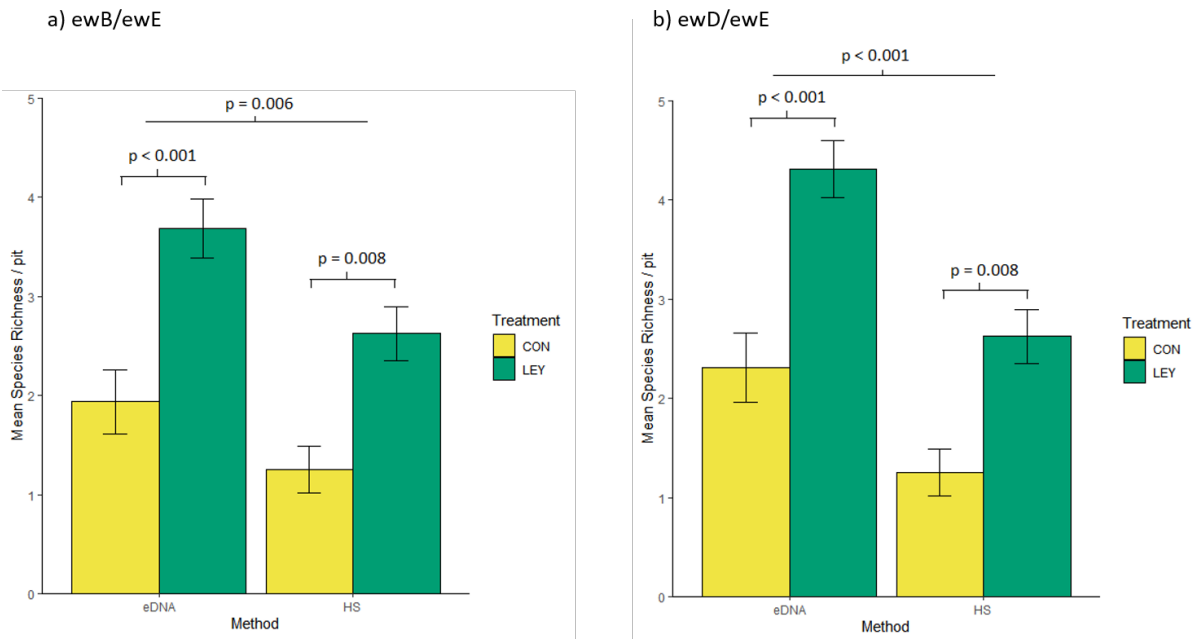


Figure 2. Mean earthworm species richness per pit (± standard error) determined using eDNA primer pair (a) ewB/ewE or (b) ewD/ewE) and traditional hand-sorting (HS). Treatment refers to land-use treatment ('con' = arable field control, 'ley' = ley strips) and p-values indicate significant differences between the methods and between land use treatments.

The species accumulation curves (Figure 3) show that, for all three methods, the number of samples was suitable to capture the earthworm diversity present and detectable using the respective method, and the levelling out of the curves indicates that additional sampling was not likely to yield more species. For traditional hand-sorting, the arable control treatment curve levelled at four species, reflecting the fact that this sampling method did not find *O. cyaneum*, *S. mammalis*, *A. caliginosa* or *L. terrestris* in the arable control treatment. Similarly, the arable curve levelled out at six species for the ewB/ewE eDNA method as *O. cyaneum* and *S. mammalis* were not detected. In contrast, all eight species of earthworm were recorded in the arable control treatments using the ewD/ewE method, although it took more samples for the arable species accumulation curve to level out when compared with the ley treatment curve. This corresponds with the species richness results, as lower species richness in the arable control treatment means that greater sampling effort is needed to find the same number of species.

Using Bray-Curtis dissimilarity calculations, NMDS ordinations successfully reached convergent solutions after 40 iterations for all three methods. With two-dimensional scaling, the reported stress levels were 0.130 for hand-sorting, 0.156 for ewB/ewE and 0.154 for ewD/ewE. Visual inspection of the NMDS ordinations suggest considerable overlap between communities belonging to both the

arable control and ley treatments (Figure 4), indicating that communities in the ley treatment were not clearly dissimilar from arable communities at this stage. This was supported by subsequent PERMANOVA tests, which did not find significant differentiation between the earthworm communities according to land use treatment (for hand-sorting pseudo-F = 1.37, p = 0.25; ewB/ewE pseudo-F = 1.33, p = 0.23; ewD/ewE pseudo-F = 2.21, p = 0.07). The land use treatment groups were also found to have homogeneous dispersions that did not differ significantly (betadisper tests, for hand-sorting p = 0.59, ewB/ewE p = 0.29, ewD/ewE p = 0.33). There was some distinction between communities as a result of field ID observed in the hand-sorting method (PERMANOVA; pseudo-F = 2.35, p = 0.02), although this was not observed by the two eDNA methods (ewB/ewE pseudo-F = 1.32, p = 0.21; ewD/ewE pseudo-F = 1.32, p = 0.19; see Supplementary Materials for NMDS plots overlaid with field ID groups).

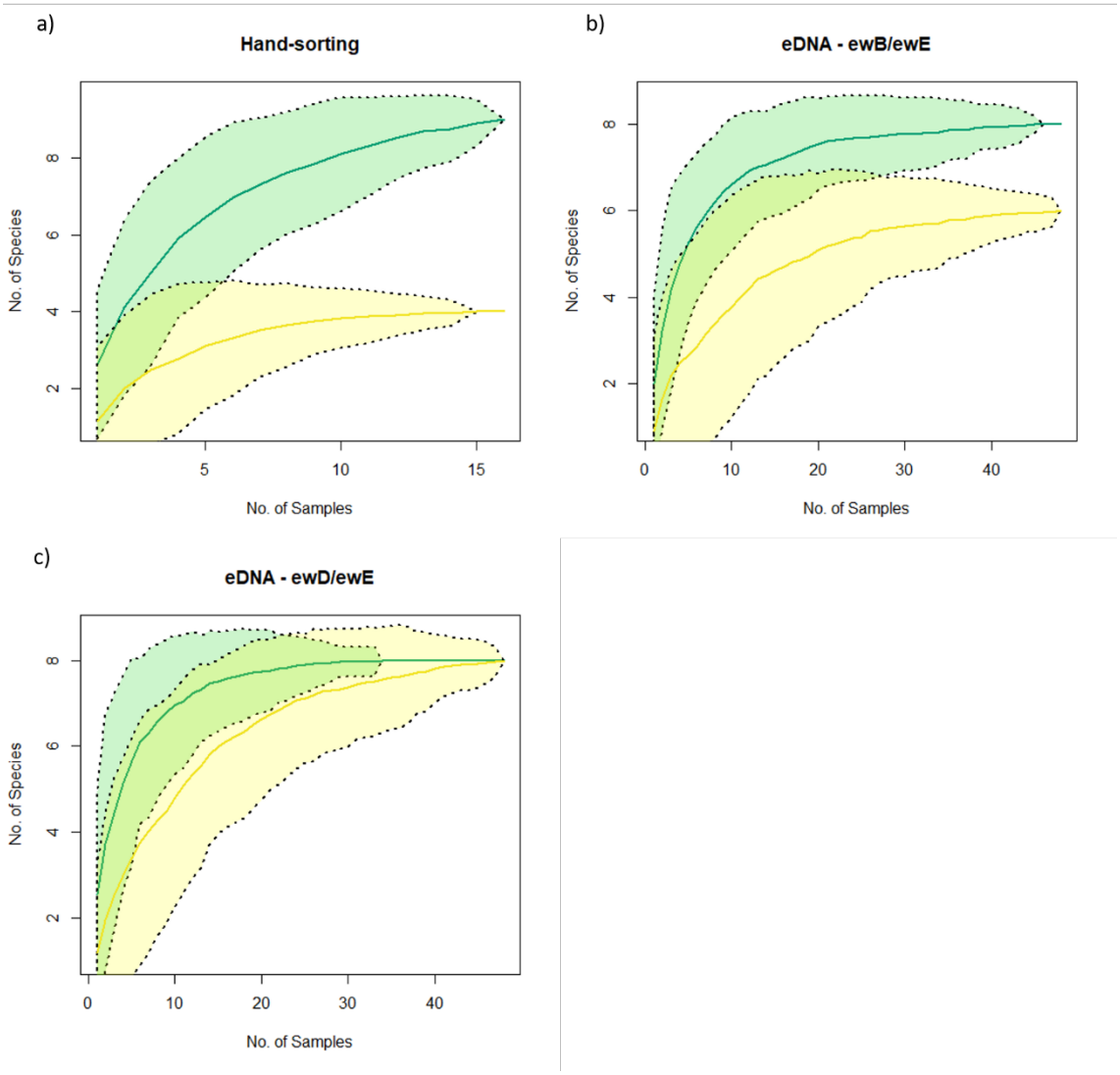


Figure 3. Species accumulation curves for (a) hand-sorting, (b) eDNA sampling using ewB/ewE primers and (c) eDNA sampling using ewD/ewE primers. Green lines represent curves for the ley treatment and yellow lines are for the arable treatment. Coloured areas around the lines with the dotted line borders indicate confidence intervals.

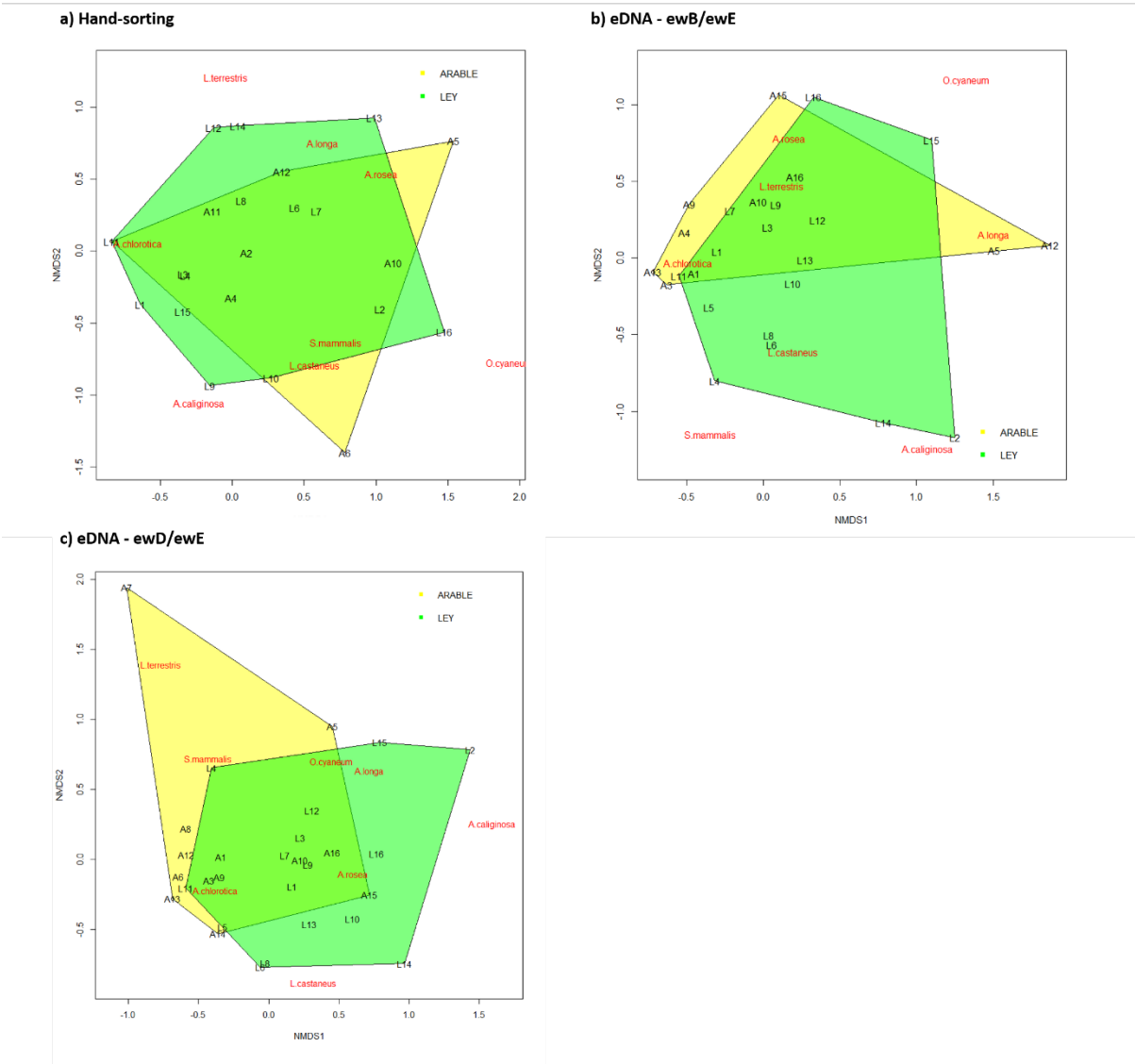


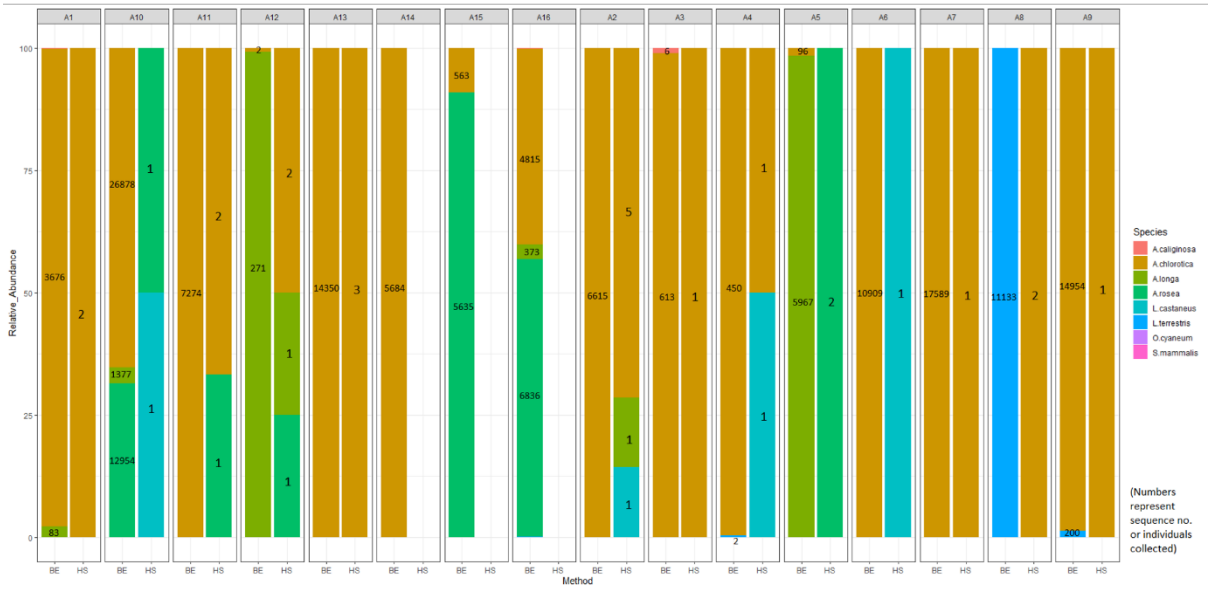
Figure 4. NMDS ordinations for (a) hand-sorting, (b) eDNA sampling using ewB/ewE primers and (c) eDNA sampling using ewD/ewE primers. The site scores in ordination space are represented by the site labels (arable sites = 'A1', 'A2' etc., ley sites = 'L1', 'L2' etc.), and the species labels are positioned at the weighted average of the site scores. The polygons connect the vertices of points made by the communities in the ley (green polygons) and arable (yellow) treatments.

Comparison of earthworm relative abundances and site occupancy proportions

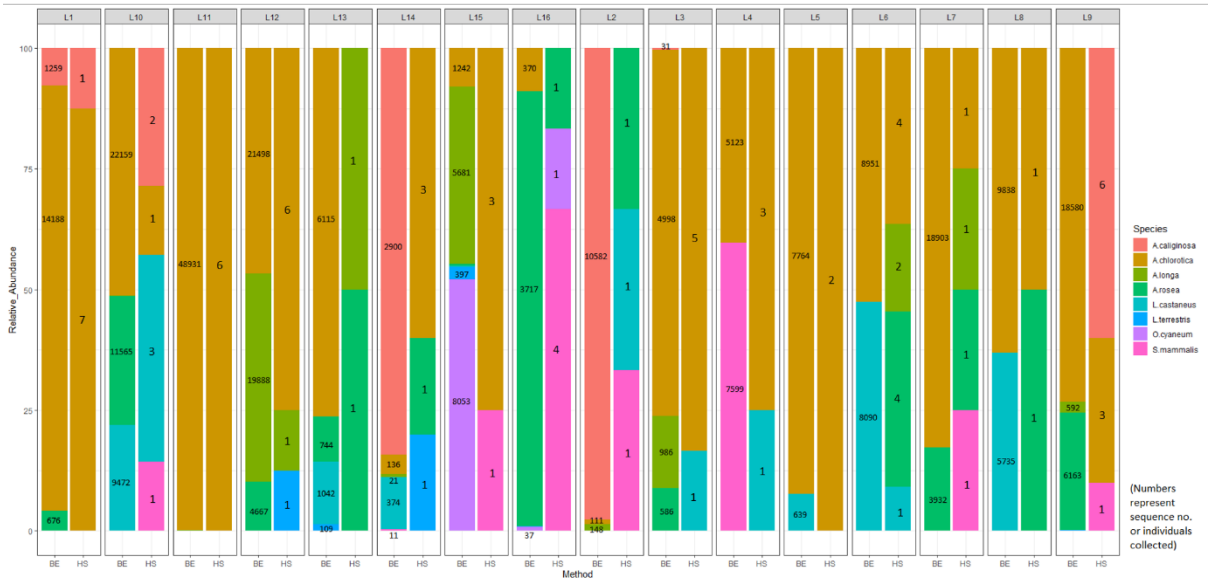
Calculating the relative abundances of species per sample revealed substantial within-sample variation when using the different methodologies (Figure 5). Plotting the relative abundances in this way does show the wider diversity patterns between the arable and ley treatments described earlier, but the variations in reported relative abundances at the sample level show that specific abundance estimations should not be inferred. At the broader scale, looking at overall site occupancy (the proportion of sites in which a given species has been found) showed slightly better consistency between the three methodologies (Table 1), but even at this broader scale variation can be seen.

Figure 5. Stacked bar charts comparing the relative abundance of species within samples, as measured by the ewB/ewE eDNA method and hand-sorting for the (a) arable and (b) ley samples. Species relative abundances are

a) ewB/ewE – Hand-sorting (Arable)



b) ewB/ewE – Hand-sorting (Ley)



marked by the different coloured bars and labelled with the actual sequence numbers and hand-sorting abundances.

Discussion

The results of this study show that environmental DNA sampling can be an effective tool for monitoring earthworm populations in agricultural ecosystems. For measuring earthworm species richness, both eDNA primer pairs performed favourably when compared with the traditional hand-sorting method and identified more species per pit sample on average. They revealed the same pattern of increased species richness in soils under ley management as was observed using the

traditional hand-sorting method, thus highlighting the beneficial effects of conversion to ley with regards to increasing earthworm diversity. The same eight earthworm species were found across all the methodologies, indicating consistent results and providing confidence that no species were missed. The species accumulation curves indicated that the level of pit sampling for both traditional hand-sorting and eDNA soil sampling was appropriate for this study system, whilst the NMDS ordinations produced for all methodologies revealed that the earthworm communities in the ley and arable treatments had not yet become clearly distinct from one another, despite the ley treatment samples having consistently higher earthworm diversity. When looking at earthworm relative abundances and the site occupancy abundance proxy across the different methodologies, both eDNA methods were found to be consistent with each other but considerable variation was seen at the sample level when compared with the hand-sorting relative abundances.

The results clearly show a positive increase in earthworm species diversity in the samples that had been converted from arable to ley management. These findings lend support to the hypothesis that conversion to ley can restore earthworm populations in arable fields that have been intensively managed, corroborating the evidence put forward by Berdeni et al. (2021) and Postma-Blaauw et al. (2012). It is interesting that, despite the higher species richness in the ley strips, the communities were not found to be distinct when analysed through NMDS and PERMANOVA. The fact that the earthworm populations in the ley strips will have been seeded by the arable field populations (as the strips were directly sown within the arable fields) and the close proximity of the two treatments may account for the similarities. Furthermore, the conversion to ley may have been too recent (33 months) to show significant divergence between the communities. In addition, some movement of earthworms between the two treatment groups cannot be ruled out, especially given the estimates of active dispersal rates that are reported in the literature for the species found here (for example Eijsackers, 2011; Butt et al., 2004; Marinissen and van den Bosch, 1992).

With regards to the performance of the different sampling methods, when compared with each other both eDNA primer pairs gave consistent results and captured similar pictures of earthworm diversity, so both could prove useful in wider-scale monitoring programmes. As predicted by Bienert et al. (2012), ewB/ewE provided better species resolution but amplified a greater proportion of *Enchytraeidae* sequences. Although we chose to focus solely on earthworms in this study, enchytraeids also offer important contributions to soils and can be good indicators of soil health (Koutika et al., 2001; Marinissen and Didden, 1997; Pelosi and Römbke, 2016), so future surveyors employing eDNA sampling may wish to utilise the ewB/ewE primers in order to include them. However, caution would be needed, as these taxa may be less well represented in the sequence

databases than earthworms. For surveys looking solely at earthworms the ewD/ewE primers may be preferable, as these amplified more earthworm sequences overall.

Both eDNA primer pairs performed well in comparison to traditional hand-sorting, identifying more species on average across all the different treatment groups and field sites. This may be because eDNA sampling is not constrained by some of the limitations of hand-sorting mentioned earlier, particularly the exclusion of juvenile worms, which cannot be identified to species level morphologically. The majority of earthworms collected through hand-sorting were juveniles (almost 83% of the total), and so this represents a significant potential reservoir of diversity that is excluded from the hand-sorting results. The lower species diversity obtained through traditional hand-sorting may also be due to worms fleeing the pit area as the soil block is excavated. Earthworms are sensitive to vibration and are known to retreat deeper into their soil burrows when disturbed, which can be an issue particularly for sampling larger vertical burrowing anecic worms whose burrows can extend well below the depth of the excavated pits (Pelosi et al., 2009; Singh et al., 2016). The site occupancy percentages reflect this lack of deep burrowing species and show that the eDNA methods were markedly better at detecting the two larger anecic species *A. longa* and *L. terrestris* (for *A.longa*, site occupancy measured by hand-sorting was 18.8% compared with 46.9% and 43.8% for the eDNA methods; for *L. terrestris* hand-sorting site occupancy was just 6.3% compared with 37.5% and 53.1% for eDNA). Hand-sorting is often combined with the use of a chemical expellant like AITC or formaldehyde, which can improve the detection rate of anecic earthworms (for example, Crittenden et al., 2015 and Holden et al., 2019), but this was deemed unfeasible in this study due to time and labour constraints.

The only species that was found to have equal or slightly lower site occupancy percentages when measured using eDNA compared with hand-sorting was the epigeic worm *S. mammalis* (6.3% and 18.8% for the eDNA primer pairs compared with 18.8% for hand-sorting). Given that all methodologies found *S. mammalis* to be relatively rare at the study site, this may simply be due to random variation or the heterogeneous spatial distribution of earthworm populations in soils (Valckx et al., 2011). However, Bienert et. al (2012) also reported underrepresentation of epigeic species by eDNA sampling. They attributed this to the limited number of samples taken and to them not taking the top few centimetres of soil where epigeic earthworms would be most active. However, although our study was not limited by these factors, we still observed the same outcome, which may indicate other possible causes. It has been shown previously that numerous factors can affect eDNA stabilisation and degradation rates in soils, including moisture levels, pH, temperature, microbial activity, soil type and chemistry (Barnes and Turner, 2016; Harrison et al., 2019; Pietramellara et al., 2009; Sirois and Buckley, 2019). Many of these attributes also vary with soil depth, so it is possible that the surface soil may experience different conditions that increase eDNA degradation rates so that surface-dwelling

species are less represented. Furthermore, it was only the longer ewB/ewE primer pair that showed lower site occupancy, and we would expect longer DNA fragments to be more susceptible to degradation. Future studies may shed light on whether the lower detection of epigeic species here is due to a random phenomenon or a result of wider eDNA sampling bias. More research is needed on how eDNA stabilisation and degradation rates may be affected by soil depth, particularly given the potential role of earthworm-mediated bioturbation in the transport of DNA molecules throughout the soil profile, as demonstrated by Prosser and Hedgpeth (2018).

Related to the issue of eDNA degradation, the capacity for eDNA to persist in the environment over time has led to ongoing debate over the extent to which eDNA sampling is measuring current or past populations (Sirois and Buckley, 2019; Thomsen and Willerslev, 2015). Our study shows clear differences in soil eDNA profiles after a few years under different management treatments and these patterns are similar to those revealed by traditional sampling techniques. This shows that eDNA sampling is sensitive enough to pick up fine-scale differences in earthworm communities in a relatively short time period, in spite of any 'background' eDNA that may be present. It could therefore be a useful tool for gauging the success of new soil management regimes that have been implemented to improve on-farm soil biodiversity.

However, we cannot definitively rule out that some of the sequences in our totals may originate from background eDNA deposited by earthworms that were no longer present. It is possible, for example, that for the ewD/ewE primer pair the small numbers of sequences of *S. mammalis* and *O.cyaneum* in the arable samples may be due to eDNA from past populations, as these species were not picked up by hand-sorting or the longer ewB/ewE primer pair (which would not have picked up shorter, more degraded eDNA fragments). This is just one potential explanation for the presence of these species in the arable controls and it is unlikely that any legacy eDNA present would significantly change the results in this case, but future research focused on the degradation rate of eDNA in soils and ways to account for background effects is essential (Thomsen and Willerslev, 2015). A recent study by Marshall et al. (2021) offers a useful starting point for this, which describes a method of estimating the age of eDNA based on accompanying eRNA sampling and analysis of the eDNA:eRNA ratio. Utilising techniques like this may prove useful if agricultural soil eDNA surveys become more widespread, as they would allow the surveyor to account for variations in eDNA degradation rates that may be brought about by different agricultural practices (Foucher et al., 2020; Sirois and Buckley, 2019).

Sampling intensity and effort is another important aspect to consider if earthworm eDNA sampling is to be taken up for use in more widespread soil monitoring schemes (Dickie et al., 2018). The species accumulation curves indicated that an appropriate level of sampling was carried out for this study

system but, for accurate sampling of larger sites and whole farms, more samples are probably needed. For developing a standardised sampling regime, future soil health monitoring programmes should conduct further investigations across multiple sites and calculate appropriate eDNA soil sample numbers per unit area, similar to the work of Valckx et al. (2011) for hand-sorting and chemical extraction. Surveys should also calculate accompanying species accumulation curves to check for appropriate coverage, as performed in this study. With regards to sampling effort, the collection of soil samples for eDNA analysis was less time and labour intensive in the field than hand-sorting, but this was traded off against more time spent in the lab preparing the samples for sequencing and subsequent bioinformatics. Future streamlining could be achieved, which could further reduce the sampling effort needed, including through the use of faster decontamination techniques in the field (for example Foucher et al., 2020) or the utilisation of labs with dedicated eDNA facilities and protocols in place.

Achieving a reliable indicator of species abundances is a key challenge that remains for soil eDNA sampling (Kestel et al., 2022). As well as diversity, earthworm abundance is very important for agricultural soil health as it affects soil functioning, how quickly earthworms can improve degraded soils and the rate at which they spread to new areas (Bertrand et al., 2015; Capowiez et al., 2014; Mathieu et al., 2010; Schon et al., 2017). For our study, inferring species abundance from the eDNA sequence numbers is problematic. Raw sequence numbers can be distorted during the PCR amplification, pooling and sequencing processes (Fonseca, 2018; Kebschull and Zador, 2015; Pinto and Raskin, 2012). A common solution in metabarcoding studies is to use relative abundance data instead, but this is also not without criticism (Jian et al., 2020; Lovell et al., 2015; Pinto and Raskin, 2012). Our results suggest that caution should be taken when trying to infer actual abundance patterns from soil eDNA sequence numbers and relative abundance data, due to high within-site variability between the eDNA measures and hand-sorting abundances. Site occupancy proportions has been suggested to be a suitable alternative proxy for abundance in eDNA studies (Hänfling et al., 2016), which is slightly more consistent with the hand-sorting abundances obtained. However, this only gives an overall abundance estimate for each species across the whole sample area, and does not give any information on abundances within and between samples. Progress is being made in developing solutions that will allow more rigorous estimation of abundances from eDNA sampling, including through the use of new statistical techniques and quantitative PCR (Lovell et al., 2015; Spear et al., 2021; Yates et al., 2019).

Conclusion

Environmental DNA sampling represents a promising alternative to the conventional ways of measuring earthworm diversity in agroecosystems that could help to increase standardisation, reduce time spent in the field and tackle some of the existing sampling biases. Our results show that eDNA can be used to sample active agricultural fields and detect fine-scale changes in earthworm diversity brought about by different management practices, making it a good candidate for use in wider soil health monitoring programmes. Future research could help to optimise and refine the technique further, by addressing some of the key unanswered questions surrounding eDNA deposition and degradation in soils, the contribution of legacy eDNA and obtaining reliable abundance information from sequence data. Based on the results of this study and the previous work described here, it is anticipated that eDNA sampling could become an important tool for monitoring earthworm diversity and soil health in agriculture, just as it has become a vital instrument for monitoring marine and aquatic habitats.

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