DNA metabarcoding of faecal samples for assessment of invertebrate communities in pasture land

A pilot project to determine the efficacy of faecal DNA analysis as a monitoring method in soil health management

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Natural England Commissioned Report NECR452

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Report details

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Citation

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Foreword

Natural England commissioned this project in partnership with the National Trust to evaluate the effectiveness of using a DNA metabarcoding approach to assess the presence/absence of dung beetles and to evaluate species diversity and community composition within livestock faeces. Results from this work will be used to determine the feasibility of using livestock faeces for DNA metabarcoding to assess local biodiversity and to consider the potential for citizen scientist to assist with sample collection.

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Executive summary

Herbivore faeces provides a vital resource for invertebrates in terrestrial landscapes and dung beetles are often considered to be good indicators of biodiversity. Here we undertake a small pilot project, using eDNA techniques, to assess feasibly of using livestock faecal samples for monitoring of dung beetles and broader invertebrate communities in pasture land in the United Kingdom. Samples were collected from pastoral land on the National Trust's Woolbeding estate.

Each faecal deposit was sampled using a standardised method, collecting replicates to assess DNA variation in subsampling. In total, 33 faecal kits were deployed, returned, and analysed. Each sample was analysed with three assays designed for invertebrate metabarcoding.

Key results from the DNA data:

- The BF2/BR2, BF2/BR3, mlCOIintF_XT/jgHCO2198 assays detected 34, 55, and 82 target taxa, respectively.
- A total of 23 arthropod taxa were identified to species level, belonging to the orders Coleoptera, Diptera, Hemiptera, Lepidoptera, Gastropoda, Collembola, and Mesostigmata.
- No dung beetles were detected.
- Three nematode species that are parasitic in livestock were also detected.
- Differences were observed in invertebrate community composition between sheep and cow faeces.

Not all samples produced data for target taxa, with each assay, with some samples not reported due to insufficient target DNA for sequencing, livestock and domestic pet contaminants, failure to pass sequence thresholds or unassigned taxa.

The between replicate variation suggests that future development of a monitoring method should include subsampling within faecal deposits to account for the potential of localised distribution of invertebrates between individual faecal deposits. The eDNA approach taken here has the potential to be developed into a standard monitoring method to efficiently provide wider invertebrate community data, to a high-level, across large scale areas, to a standardised degree.

Contents

Introduction

Herbivore faeces provides a vital resource for invertebrates in terrestrial landscapes. The associations of over 400 species of British insects are supported, either directly (e.g. food source) or indirectly (e.g. predatory) across cattle, sheep, deer and horse faeces (Skidmore, 1991). Dung beetles are of particular interest as ecological indicators in agricultural environments (Filgueiras and others., 2015), with species richness, diversity and abundance of the taxa found to trend with habitat and grazing intensity (Buse and others., 2015; Tonelli and others., 2018).

Traditional methods to monitor faecal communities are labour and expertise intensive, and often inefficient. Recently, non-invasive environmental DNA (eDNA) metabarcoding has been trialled for biomonitoring of faecal-associated invertebrates (Sigsgaard and others., 2021). Results were promising, with several functional groups represented, and ecological associations evident. Here we undertake a small pilot project, using similar eDNA techniques, to assess feasibly of using livestock faecal samples for monitoring of dung beetles and broader invertebrate communities in pasture land in the United Kingdom. Success in the project could demonstrate the propensity for faecal invertebrate DNA surveys for monitoring impacts of soil management practices and regenerative farming, leading to increased soil biodiversity.

The West Sussex Arun-Rother river basin has in recent years suffered from serious soil erosion issues and there has been a catchment sensitive farming initiative in place to resolve some of the issues associated with intensive agricultural practices. Efficient detection of dung beetles and other invertebrates in animal faeces was felt to be an effective way to measure improvement to overall soil health and biodiversity. In addition, the surrounding mosaic of woodland, hedgerows and pasture, with higher insect biomass, might benefit a range of bat and bird species. In particular the Greater horseshoe bat has recently been found breeding at a maternity roost within 10Km of the Woolbeding site. This is the first know maternity roost of this species in south east England for over 100 years and dung beetles are key element of this bat's summer diet. Improved detection of dung beetle populations might therefore benefit habitat suitability modelling for Greater horseshoe bats.

Project objectives

The overall objective is to trial the use of eDNA analysis on livestock faeces from a National Trust farm in the West Sussex project area to test the following:

- The effectiveness of the testing system to assess presence/absence of dung beetles in areas of cattle and possibly sheep/deer grazed pasture and wood pasture

- The effectiveness of the system in assessing dung beetle and/or invertebrate community composition and species diversity
- Does this pilot project provide a model for future testing going forward, especially if NE and partner NGOs have staff and volunteers who can efficiently collect samples to ensure we have a citizen science element (collection rather than analysis) in the future.

Methods

Sampling Locations

Sampling was undertaken by NatureMetrics staff at Woolbeding farmland, part of the wider National Trust Woolbeding countryside, on the 17th of September 2021. The countryside estate sits within the South Downs National Park and encompasses Woolbeding and Pound Commons, a biological Site of Special Scientific in West Sussex, and approximately 900 hectares of grazed land. Habitat and soil types include heathland with free draining sandy soils (acidic); low input and permanent pasture with free draining sandy/loamy soil (acidic); and chalk grassland with shallow lime rich soil.

Three breeds of cattle are present on the Estate: Belted Galloway, Long Horn and Sussex. Herdwick sheep are also kept for conservation grazing purposes. One cow faecal deposit was sampled on the lowland heathland and all remaining samples were collected from cattle and sheep faeces on the permanent pasture (Figure 1).

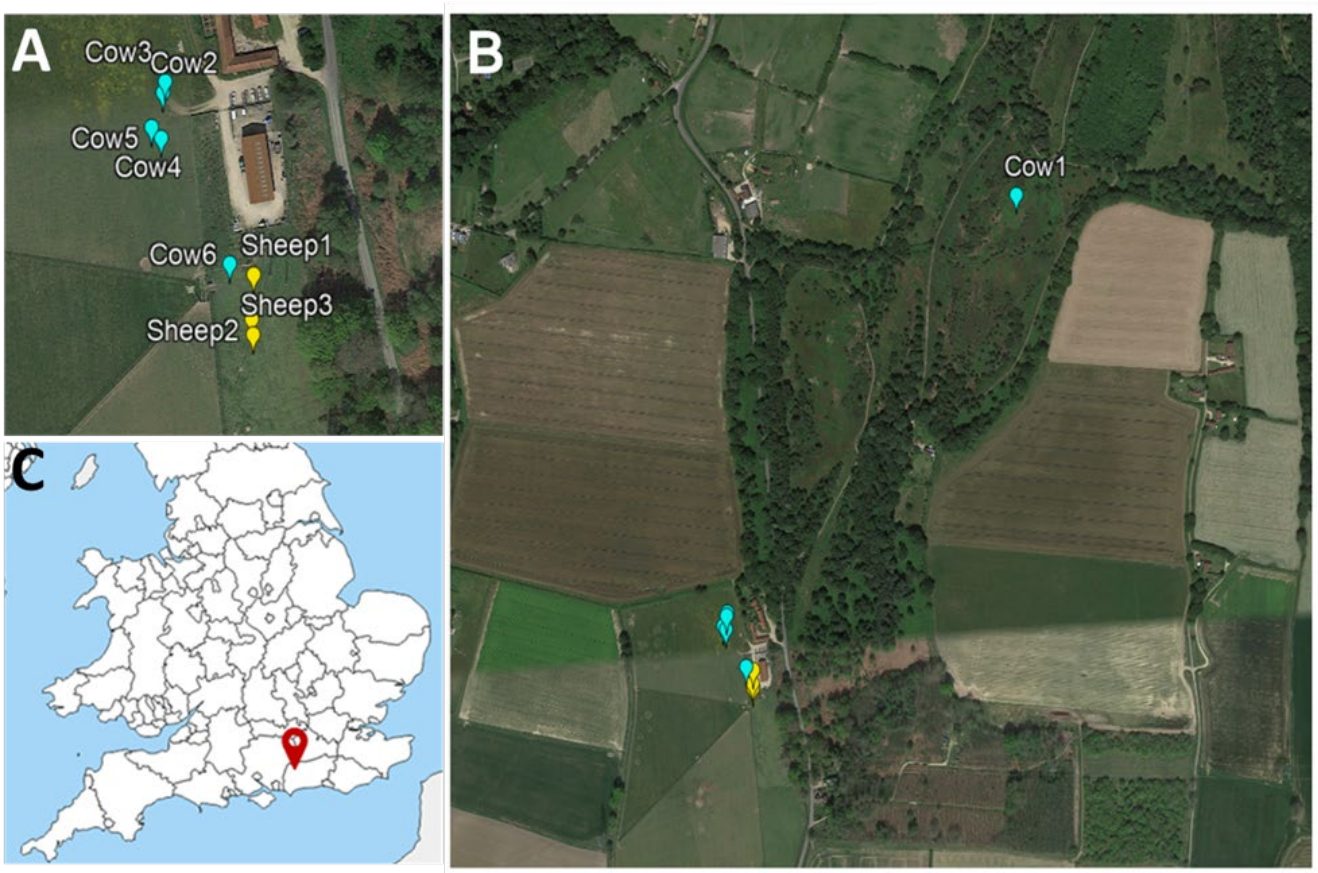

Figure 1: Sampling locations for the project showing A) cow samples 2-6 and sheep samples 1-3, B) cow sample 1 in relation to the other samples and C) the location of the sampling area. Google earth V 7.3.4. (September 14, 2019). England, United Kingdom. 50° 59’ 29.98”N, 0° 44’ 43.21”W, Eye alt 11.39 km. Map data ©2019 Google [July 22, 2022].

Sampling Method

In total, 33 samples were collected using NatureMetrics prototype Species from Faeces kits. Each kit consisted of sterile sampling tools, obtaining sample matter of at least 1 mL in volume, in a 50 mL sterile tube with 10 mL of preservation buffer. Six cow faecal deposits were sampled, with five separate samples collected per deposit (Figure 2) to assess variability within a single deposit, resulting in a total of 30 samples of cow faeces. Three sheep faecal deposits were sampled but replicates were not collected from each deposit. Instead, multiple pellets were added to the same kit to give one composite sample per deposit. Samples were transported at ambient temperature then stored at -20°C until further processing. Sample meta-information is given in Table 1, along with any visual evidence of possible dung beetle activity, and any incidental occurrences of invertebrates observed during sampling.

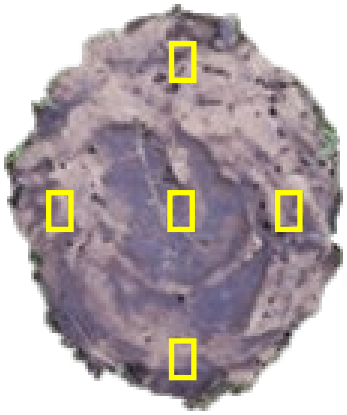

Figure 2: Indicative replicate sampling locations for each cow faecal deposit. Image by Kate Denton.

Table 1: Sample information and field notes. See Appendix A for photographs of each sample.

Sample ID	Animal	Habitat	Approx. dimensions of whole sample (l x w x h) (cm)	No. of samples taken	Evidence of potential dung beetle holes in faeces?	Other evidence
Cow1	Cow	Heathland	23 x 15 x 7.5	5	No	Isopoda, Arachnida
Cow2	Cow	Permanent pasture	18 x 12.5 x 2	5	No	Unidentified larvae, unidentified invertebrates
Cow3	Cow	Permanent pasture	25.5 x 14 x 1.2	5	No	None
Cow4	Cow	Permanent pasture	33 x 25.5 x 5	5	Yes	Coleoptera
Cow5	Cow	Permanent pasture	23 x 18 x 6.5	5	Yes	Coleoptera, Diplopoda, Diptera
Cow6	Cow	Permanent pasture	34 x 30 x 6.5	5	Yes	Coleoptera
Sheep1	Sheep	Permanent pasture		1	No	
Sheep2	Sheep	Permanent pasture		1	No	

Sample ID	Animal	Habitat	Approx. dimensions of whole sample (l x w x h) (cm)	No. of samples taken	Evidence of potential dung beetle holes in faeces?	Other evidence
Sheep3	Sheep	Permanent pasture		1	No	

Lab analysis

Decontamination procedures

NatureMetrics have dedicated ultra-clean laboratories with a unidirectional workflow between labs for distinct stages of the eDNA metabarcoding workflow, from sample receipt to sequencing. Work was undertaken within dedicated laminar flow hoods, with ChemGene cleaning taking place after each use, and a regular weekly deep cleaning schedule. Equipment was cleaned using DNA decontamination wipes. Laminar flow hoods were UVC sterilised prior to setup and operated with air flow turned off to avoid contamination. All PCR preparation is conducted in a pre-PCR clean room within a PCR hood that is ChemGene and UVC treated before and after any PCR set-up.

DNA extraction

DNA from faecal samples was extracted using a DNeasy 96 PowerSoil Pro QIAcube HT Kit, following manufacturer's protocol. An extraction blank, where no faeces are added to the DNA extraction, was also processed for each extraction batch. Extractions were performed in three batches. DNA was quantified using a Qubit dsDNA Broad Range Assay Kit (Thermo-Fisher Scientific) following extraction.

PCR amplification

Three replicate PCRs for each sample and extraction blank were amplified with three primer sets designed for invertebrate metabarcoding and targeting the mitochondrial cytochrome c oxidase subunit I (COI) gene (See Table 2 for details and references).

Table 2: Invertebrate COI primer sets used in this study

Primers (forward/reverse)	Marker	Reference
BF2/BR2	COI	Elbrecht & Leese 2017
BF3/BR2	COI	Elbrecht and others. 2019/Elbrecht & Leese 2017
mlCOIintF_XT/jgHCO2198	COI	Leray and others. 2013/Wangensteen and others. 2018

The BF2/BR2 PCR reaction contained DreamTaq Green PCR Master Mix (Thermo Scientific), 0.5 µM each primer, 0.8 mg/ml bovine serum albumin (Thermo Scientific), 1µl template DNA, and molecular grade water. The PCR cycle consisted of an initial denaturation at 94°C for 3 min, 35 cycles of: 94°C for 30 sec, 46°C for 30 sec, 72°C for 1 min, followed by a final extension at 72°C for 5 min.

The BF3/BR2 PCR reaction contained DreamTaq Green PCR Master Mix (Thermo Scientific), 0.5 µM each primer, 1µl template DNA, and molecular grade water. The PCR cycle consisted of an initial denaturation at 94°C for 5 min, 30 cycles of: 95°C for 30 sec, 48°C for 30 sec, 72°C for 50 sec, followed by a final extension at 72°C for 5 min.

The mlCOIintF_XT/jgHCO2198 PCR reaction contained DreamTaq Green PCR Master Mix (Thermo Scientific), 0.3 µM each primer, 0.8 mg/ml bovine serum albumin (Thermo Scientific), 1µl template DNA, and molecular grade water. The PCR cycle consisted of an initial denaturation at 95°C for 10 min, 35 cycles of: 94°C for 1 min, 45°C for 1 min, 72°C for 1 min, followed by a final extension at 72°C for 5 min.

All PCRs were performed in the presence of a negative control adding molecular grade water instead of DNA sample to check for reagent contamination or sample cross contamination. Success of the amplifications were confirmed by gel electrophoresis. Lab negative controls did not produce any quantifiable DNA on amplification and were not progressed to sequencing.

All positive PCR replicates were pooled and purified using MagBind TotalPure NGS (Omega Biotek) magnetic beads. A sequencing library was prepared from the purified amplicons using a combinational dual index approach based on a two-step PCR protocol (Illumina 16S Metagenomic Sequencing Library Preparation protocol from Illumina Part # 15044223 Rev. B). Amplification of the indexed PCR products were confirmed by gel electrophoresis. The indexed PCR products were purified using Mag-Bind TotalPure NGS (OMEGA BIOTEK) magnetic beads. The purified index products were quantified using a Qubit dsDNA Broad Range Assay Kit (Thermo-Fisher Scientific), normalised and pooled in equimolar concentrations.

Sequencing

Samples were sequenced on an Illumina MiSeq platform (V3 600 cycle sequencing kit) with a 20% PhiX spike in following the manufacturer's recommended specifications.

Bioinformatics

Bioinformatic processing

Bioinformatics processing was performed using a custom NatureMetrics bioinformatics data processing and taxonomic identification pipeline that builds on bcl2fastq, cutadapt and usearch. Briefly, the pipeline converts MiSeq tile image data into per-sample FASTQ

format. Paired-end FASTQ files were merged to provide a single sequence for each pair of sequencing reads. The merged reads were then primer clipped to remove the first round PCR primer sequences and length-filtered to return sequences within the length distribution of each marker gene. Reads were then quality filtered, dereplicated, denoised and clustered into OTUs at 97% (sequences flagged as chimeras were excluded). An OTU-by-sample table was generated by mapping all dereplicated reads for each sample to the OTU representative sequences.

The resulting OTUs were assigned taxonomic labels using sequence similarity searches against two reference databases, the National Centre for Biotechnology Information (NCBI) *nt* database, and the Barcode of Life Database (BOLD). The identification associated with each hit was converted to match the Global Biodiversity Information Facility (GBIF) taxonomic backbone to allow results from different databases to be combined. Assignments were made to the lowest possible taxonomic level where there was consistency in the matches, with identifications based on fewer than three reference matches flagged in the OTU-by-sample table. Minimum similarity thresholds of 98%, 95%, and 92% were used for species-, genus- and higher-level assignments respectively. Assigned taxa were checked against GBIF occurrence records for presence in the UK and elevated to higher taxonomic levels if there were no occurrences.

Thresholds were set on a per-marker basis to remove stochastic noise from the dataset as a percentage of reads observed per sample (<0.025% (mlCOIintF_XT/jgHCO2198 and BF3/BR2), <0.05% (BF2/BR2), or <10 reads, whichever is the greater threshold for the sample for the respective markers). Unidentified and non-target OTUs (i.e. OTUs identified as Chordates and/or non-Animalia taxa) have been excluded from further analysis. Domestic pets are excluded from reporting as it is impossible to assign them a taxonomy that is separate from their wild relatives. Human sequences are not reported as they are ubiquitous in the sampling and laboratory environment.

Reference libraries

The “nt” DNA nucleotide library provided by the NCBI, and BOLD were the primary source for taxonomy classification. Sequences were classified as targets of the assay, non-targets, environmental contaminants (livestock, human, domestic pets), or unclassified (non-specific amplification, and sequences with no taxonomic assignment). Species identifications were curated to ensure that there are observations within the country. Where ambiguous species identifications were observed (such as two species being equally likely to be assigned to the same DNA sequence), these were elevated to the taxonomic level where there was consensus between the two potential taxonomic identities by the curation and annotation team.

QA process

All datasets were reviewed by the Lead Bioinformatician (Product Delivery) at NatureMetrics to ensure consistency and accuracy of datasets. This included reviewing

taxonomic identifications and ensuring appropriate analysis parameterisation including OTU clustering thresholds and read-depth cut-offs.

Results

Overview

The data underlying the results is presented in Appendices A-C which are provided in a separate Excel file which accompanies this report. The sequencing count data for target taxa sequences are provided in separate sheets for each assay. The lab QC data for each sample (DNA extraction and library quantification and sample name) are presented in Appendix D within the same Excel file. All assay data include livestock as contaminant sequences. As common molecular biology reagents contain material of animal origin, which cannot be guaranteed to be DNA-free, livestock are routinely excluded after sequence processing, but are presented here. It should be noted though that it is difficult to determine whether an observed OTU is derived from the sample or from the reagents.

Of the 33 kits used 33 samples were successful across two of the assays, amplifying in all PCR replicates. For the BF3/BR2 assay, four samples failed to produce bands after PCR amplification across all replicates (1.2, 3.2, 5.3, 6.1). The BF2/BR2 assay failed to detect any target taxa in 3 samples (2.1, 2.3, 2.C). The BF3/BR2 assay failed to detect any target taxa in 4 samples (1.4, 2.1, 2.C, 5.C). The mlCOIintF_XT/jgHCO2198 assay detected target taxa in all samples. No sequencing reads were observed in the extraction blanks.

A summary of the assay results generated, and the analysis outcome is provided in Table 3. The information on the success/failure of each sample in progressing to sequencing for each assay is provided in Appendix E with the analysis outcome provided. Our standard threshold of target reads required per sample is 1000, however, to inform the feasibility of the method we have not removed any samples based on number of target reads. No normalisation of read numbers, such as rarefying, was performed due to the high number of samples with low numbers of target reads. It should be noted that by keeping in samples with low numbers of reads and not normalising read numbers, these are both likely to have an influence on subsequent comparisons of sample composition and richness.

Dung beetles were not detected in any sample with any assay. A list of the taxa detected to species level across all assays, their general association with dung and that of cattle or sheep are presented in Table 4. 24 of the 26 species detected have been previously associated, either directly or indirectly with cattle faeces (Skidmore, 1991). *Cercyon quisquilius* is the only Coleoptera taxon to be identified to species level.

The diversity of taxa recorded in cattle faeces is considerably broader when compared to that of sheep (Appendix F). The drastically reduced number of samples and replicates in

the latter restrict the relevance of this result. Of particular note though is the lack in diversity of taxa belonging to the Diptera order in sheep samples.

Table 3: Summary of samples by assay and analysis outcome

Analysis outcome	BF2/BR2	BF3/BR2	mlCOIintF_XT/ jgHCO2198
Samples with target taxa – sequenced and reported	30	25	33
Samples which failed troubleshooting prior to sequencing – not sequenced or reported	0	4	0
Samples with only environmental contaminant sequences – sequenced but not reported	0	0	0
Samples with only environmental contaminant sequences and unassigned taxa – sequenced but not reported	0	4	0
Samples with only unassigned taxa – sequenced but not reported	0	0	0
Samples with target reads less than 10,000	33	29	32
Samples with target reads less than 1000	22	16	16
Samples with target reads less than 100	7	8	0

Table 4: List of taxa detected to species level with eDNA analysis of cow and sheep faecal samples, and their association to dung. Some cells have been intentionally left blank.

Higher taxa	Species	Dung-associated	Previously reported in cattle[1]	Previously reported in sheep[1]
Mesostigmata	*Macrocheles glaber*	Indirectly		
Collembola	*Lepidocyrtus cyaneus*	Indirectly		
Collembola	*Desoria grisea*	Indirectly		
Collembola	*Folsomia quadrioculata*	Indirectly		
Coleoptera	*Cercyon quisquilius*	Yes	x	
Diptera	*Sylvicola punctatus*	Yes	x	
Diptera	*Hylemya vagans*	Yes	x	
Diptera	*Pegoplata infirma*	Yes		
Diptera	*Monardia toxicodendri*	Yes	x	
Diptera	*Camptocladius stercorarius*	Yes	x	
Diptera	*Azelia cilipes*	Yes	x	
Diptera	*Mesembrina meridiana*	Yes	x	
Diptera	*Morellia simplex*	Yes	x	
Diptera	*Psychoda phalaenoides*	Yes	x	
Diptera	*Sepsis duplicata*	Yes	x	
Diptera	*Sepsis flavimana*	Yes	x	
Diptera	*Sepsis thoracica*	Yes	x	
Diptera	*Coproica lugubris*	Yes	x	x
Diptera	*Lotophila atra*	Yes	x	x
Diptera	*Sargus flavipes*	Yes	x	
Hemiptera	*Rhopalosiphum padi*	No		
Lepidoptera	*Blastobasis adustella*	No		
Gastropoda	*Arion intermedius*	Indirectly		
Nematoda	*Cooperia onchophora*	Indirectly	x	
Nematoda	*Ostertagia ostertagi*	Indirectly	x	
Nematoda	*Teladorsagia circumcincta*	Indirectly		x

1. SKIDMORE, P., 1991. Insects of the British cow-dung community. Field Studies Council.

BF2/BR2 Assay

The BF2/BR2 assay detected 34 target OTUs across all the samples and they all belonged to one phylum (Arthropoda). The minimum taxon richness was 1 (Cow2.2, Cow5.3, Cow5.4, Cow6.1, Cow6.C) and the maximum taxon richness was 7 (Cow1.1, Cow1.4, Cow4.3). The OTU with the highest proportion of reads across samples was from the bee family Apidae and was detected in 11/33 samples. No taxa were detected in all samples.

Of the OTUs that were identified to species level, the taxa with the greatest proportion of sequencing reads across the samples were a slender springtail (*Lepidocyrtus cyaneus*), a non-biting midge (*Camptocladius stercorarius*), and a Sepsidae fly (*Sepsis flavimana*), and the taxa with the highest frequency of detections were *Lepidocyrtus cyaneus* and *Camptocladius stercorarius* (both present in four samples).

The invertebrate community compositions of the sheep samples were too divergent from the cow samples to constrain within the axes of NMDS. Within the cow samples, some differences were observed among the samples (Figure 3). The replicates from Cow 3 were more variable than those within the other faecal deposits. The taxa assemblages of Cow 1 appear to be divergent from the rest of the samples and subsamples. The 95% confidence intervals of Cow 5 and Cow 6 overlap considerably. With the exception of subsample Cow 6.2, the compositions of Cow 6 appear to cluster within those of Cow 5. Cow 3 and Cow 4 also appear to be more similar to each other than the other samples.

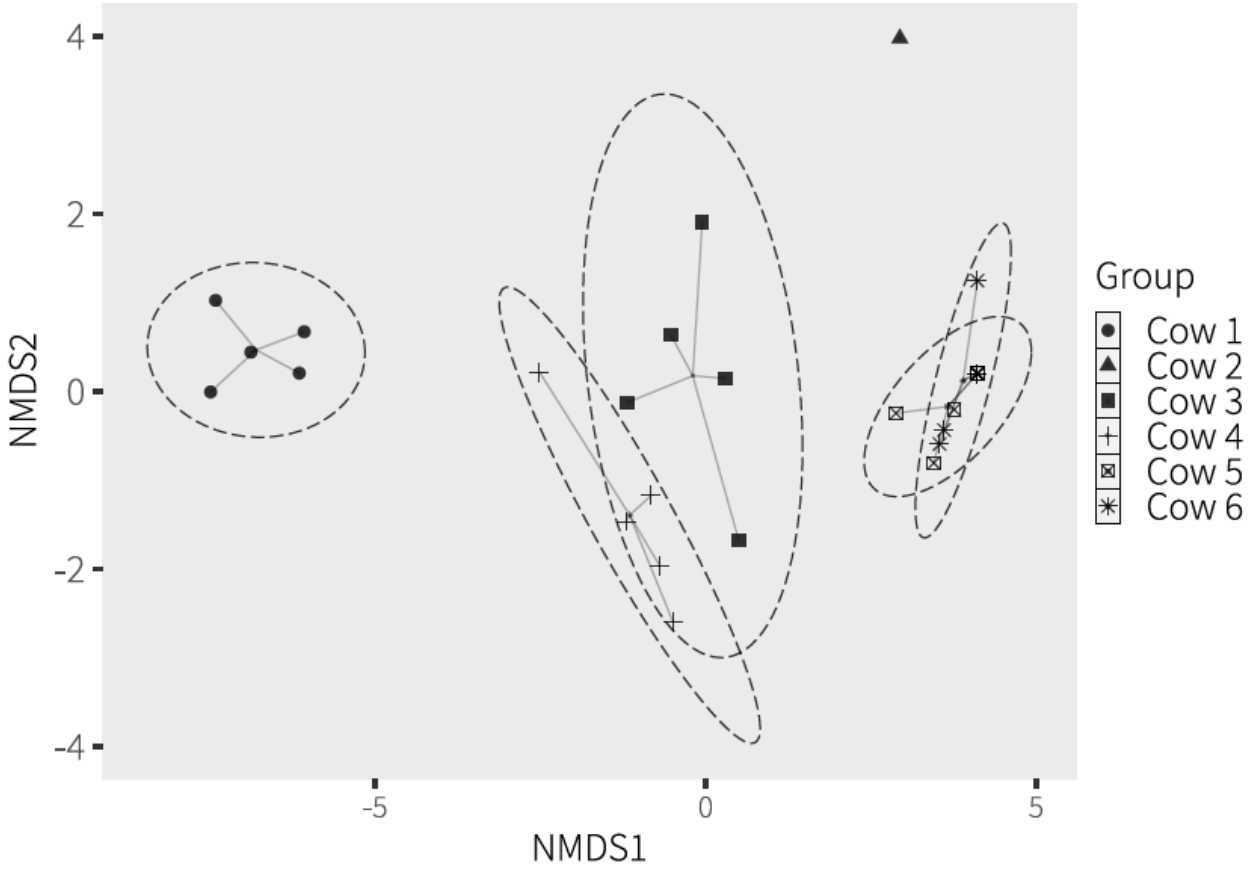

Figure 3: NMDS ordination plot based on Jaccard similarity index for target taxa in cow faeces using the BF2/BR2 assay. Point shape denotes Dung ID. 95% confidence intervals for each faecal deposit are indicated by dashed ellipses. Replicates collected from the same Dung ID are connected with a solid line. Cow2.2 and all sheep samples are excluded from this plot.

A bubble plot of the assay's target detections across all successful samples is provided in Figure 4. Cow samples are dominated by class Insecta, in comparison to Arachnida and Collembola in Sheep samples. For the cow originating samples, Coleoptera sp. (Cow 1),

Apidae sp. (Cow 3 and Cow 4) and Thysanoptera (Cow 5 and Cow 6), characterise the samples.

Cow 3 and Cow 4 faecal deposits had the highest cumulative taxon richness, but Cow 1 had the highest median richness per replicate (Figure 5). Cow 6 showed the least variation in richness among replicates.

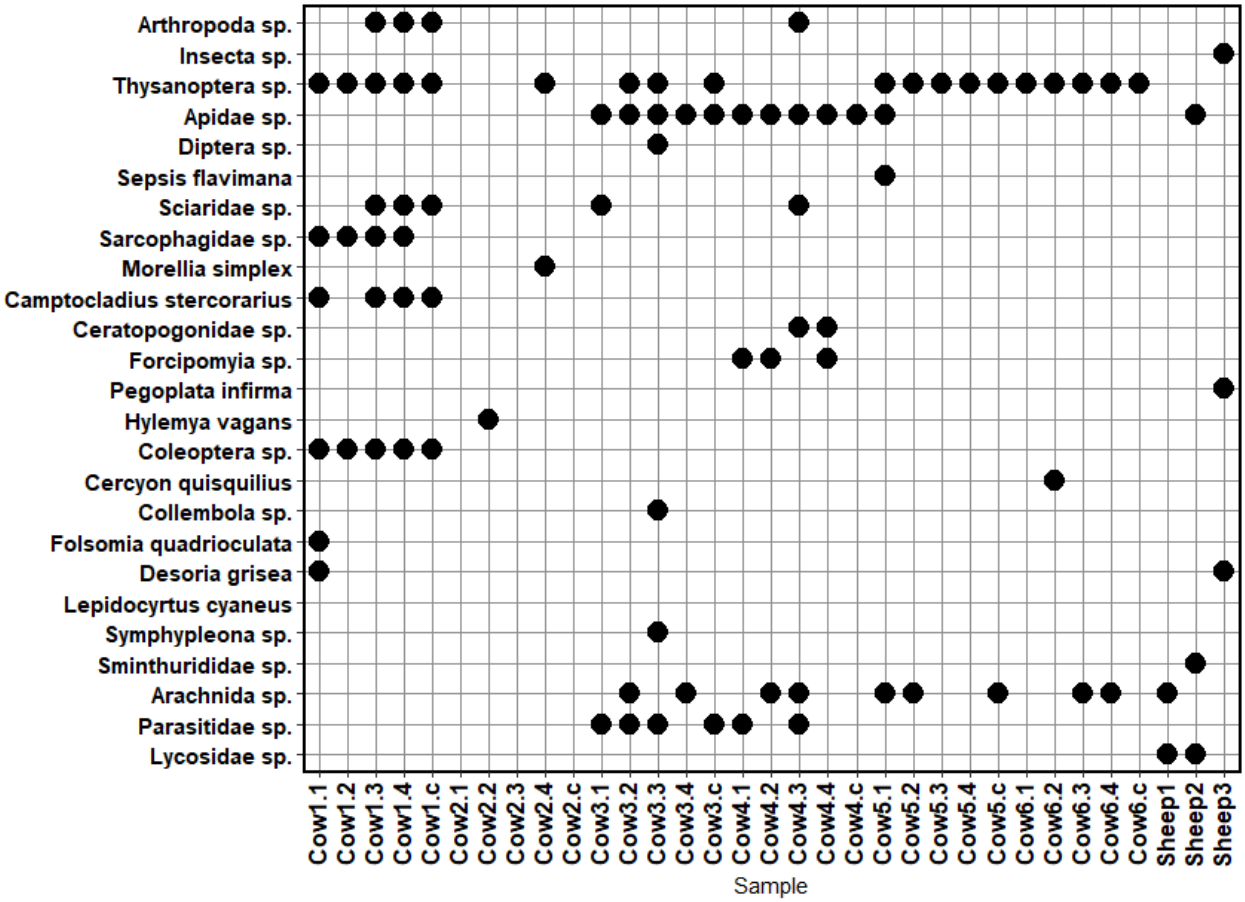

Figure 4: The different taxa (rows) within each sample (columns) detected using the BF2/BR2 assay. Black dots indicate the presence of each taxon in a sample.

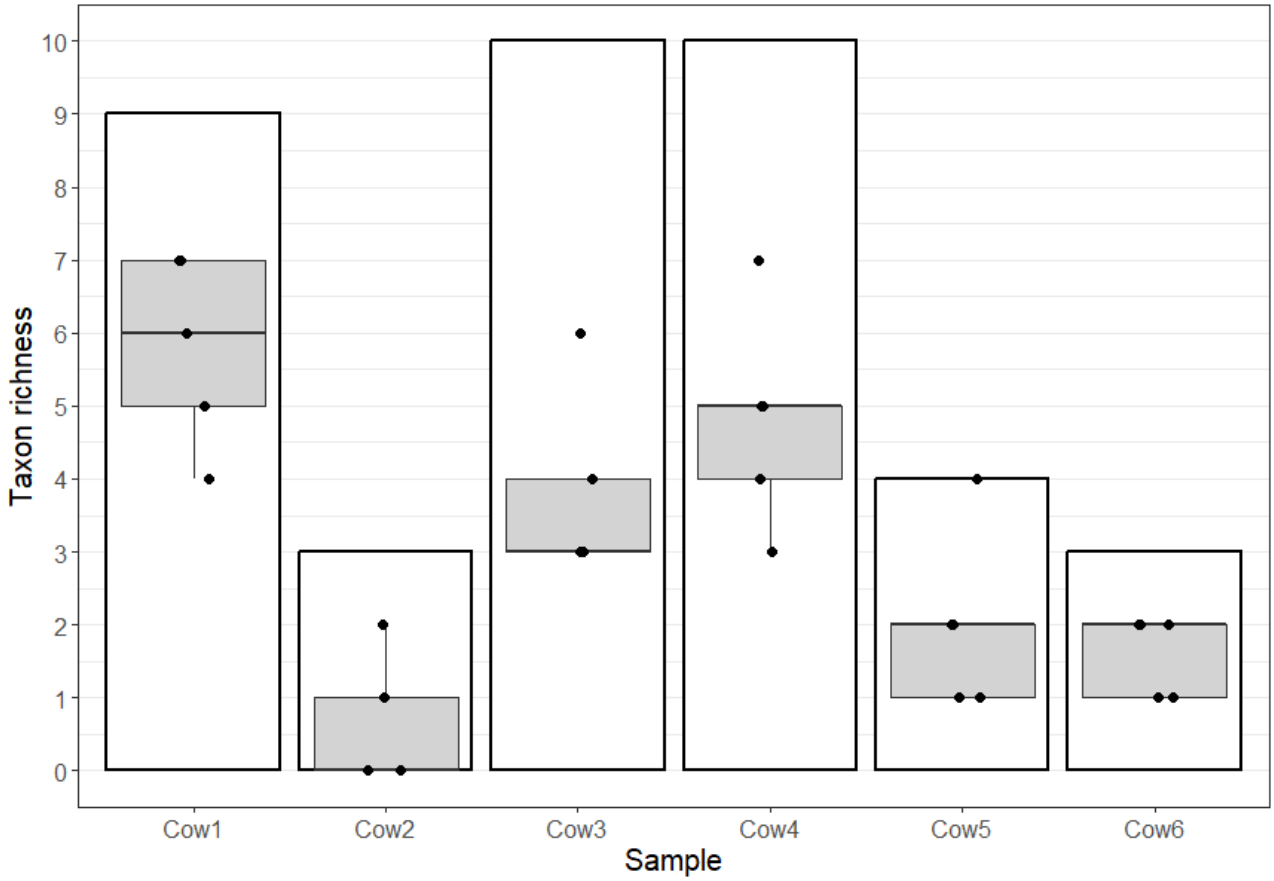

Figure 5: Taxon richness (number of OTUs) for invertebrate communities within each cowpat detected using the BF2/BR2 assay. The bar shows cowpat-level taxon richness (cumulative richness for each cowpat). The boxplot shows subsample-level richness, with the box depicting the median between the upper/lower quantiles, the whiskers indicating minimum and maximum values, and dots showing richness values of each sample. Any samples beyond the whiskers are considered as outliers which are 1.5x the interquartile-range away from the upper or lower quartile.

BF3/BR2 Assay

The BF3/BR2 assay detected 55 target OTUs belonging to two phyla (Arthropoda and Nematoda) across all the samples. The minimum species richness was 1 (Cow2.3, Cow2.4, Cow6.C, Sheep1). The maximum species richness was 12 (Sheep3). The OTU with the highest proportion of reads across samples was from the bee family Apidae, which was detected in 12/25 samples. No taxon was detected in all samples, but one OTU from the Wolf spider family Lycosidae was detected in all sheep faecal samples.

Of the OTUs that were identified to species level, the taxa with the greatest proportion of sequencing reads across the samples were a species of slender springtail (*Lepidocyrtus cyaneus*), the Bird cherry-oat aphid (*Rhopalosiphum padi*), and a species of root-maggot fly (*Hylemya vagans*), and the taxa with the highest frequency of detections were a Sepsidae fly (*Sepsis flavimana,* detected in four samples), a species of slender springtail

(*Lepidocyrtus cyaneus*, three samples), the Bird cherry-oat aphid (*Rhopalosiphum padi,* three samples), a species of root-maggot fly (*Pegoplata infirma,* three samples), and a species of lesser dung fly (*Coproica lugubris*, three samples).

Species of note detected by the assay included the brown stomach worm (*Teladorsagia circumcincta*), a parasite of sheep and goats.

The invertebrate community compositions of all the sheep samples and Cow 2 were too divergent to constrain within the axes of the NMDS. Within the remaining cow samples, there is partial clustering whilst maintaining some within sample/between sample dissimilarity (Figure 6). Cow 3 and Cow 6 show the greatest variability within replicates from the same faecal deposit. The taxa assemblages of Cow 1 appear to be divergent from the rest of the samples. The 95% confidence intervals of both Cow 5 with Cow 6 and Cow 3 with Cow 4 partially overlap, suggesting similarity in community taxa.

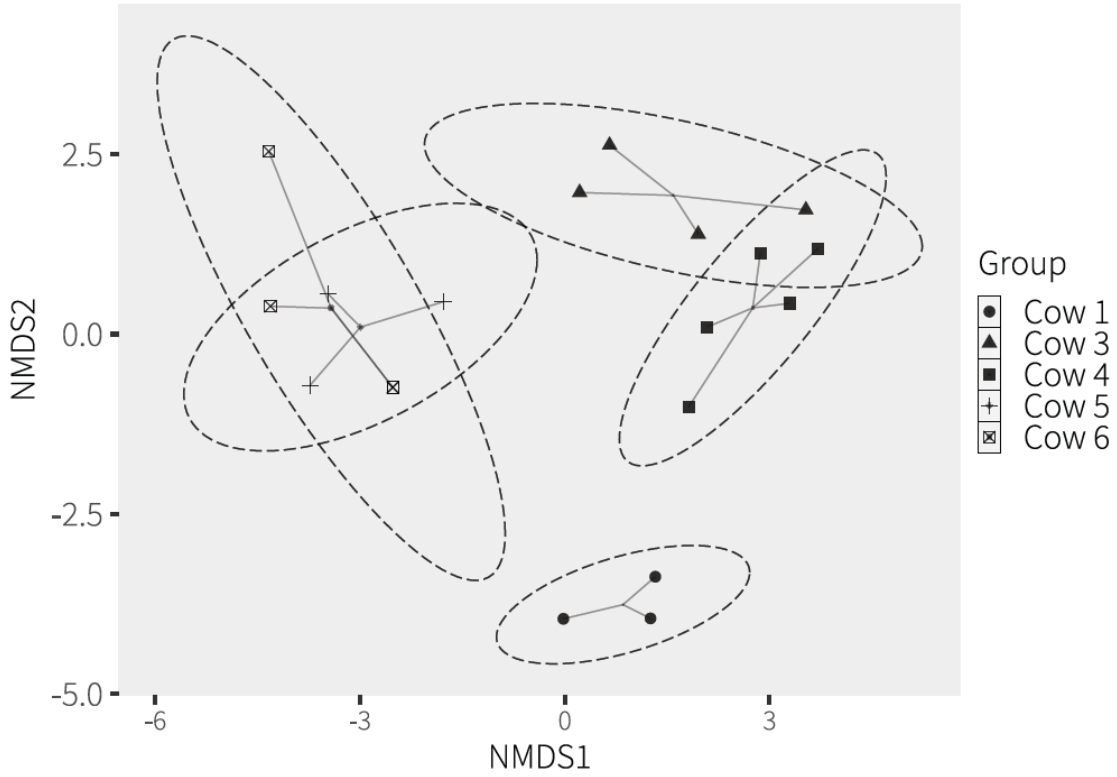

Figure 6: NMDS ordination plot based on Jaccard similarity index for target taxa in cow faeces using the BF3/BR2 assay. Point shape denotes Dung ID. 95% confidence intervals for each faecal deposit are indicated by dashed ellipses. Replicates collected from the same faecal deposit are connected with a solid line. The sheep samples and Cow2.2, Cow2.3 and Cow2.4 were excluded from the plot.

A bubble plot of the assay's target detections across all successful samples is provided in Figure 7. Cow samples contain predominantly class Insecta. For the cow originating samples, Coleoptera sp. (Cow 1), Apidae sp. and Parasitidae sp. (Cow 3 and Cow 4) and Thysanoptera (Cow 5 and Cow 6), characterise samples. Sheep1 is characterised by

Lycosidae sp., with assignments in the two remaining sheep samples being split between Arachnida, Collembola and Insecta.

Cow 4 faecal deposit had the highest cumulative taxon richness, but Cow 1 had the highest median richness per replicate (Figure 8). Cow 3 showed the least variation in richness among replicates.

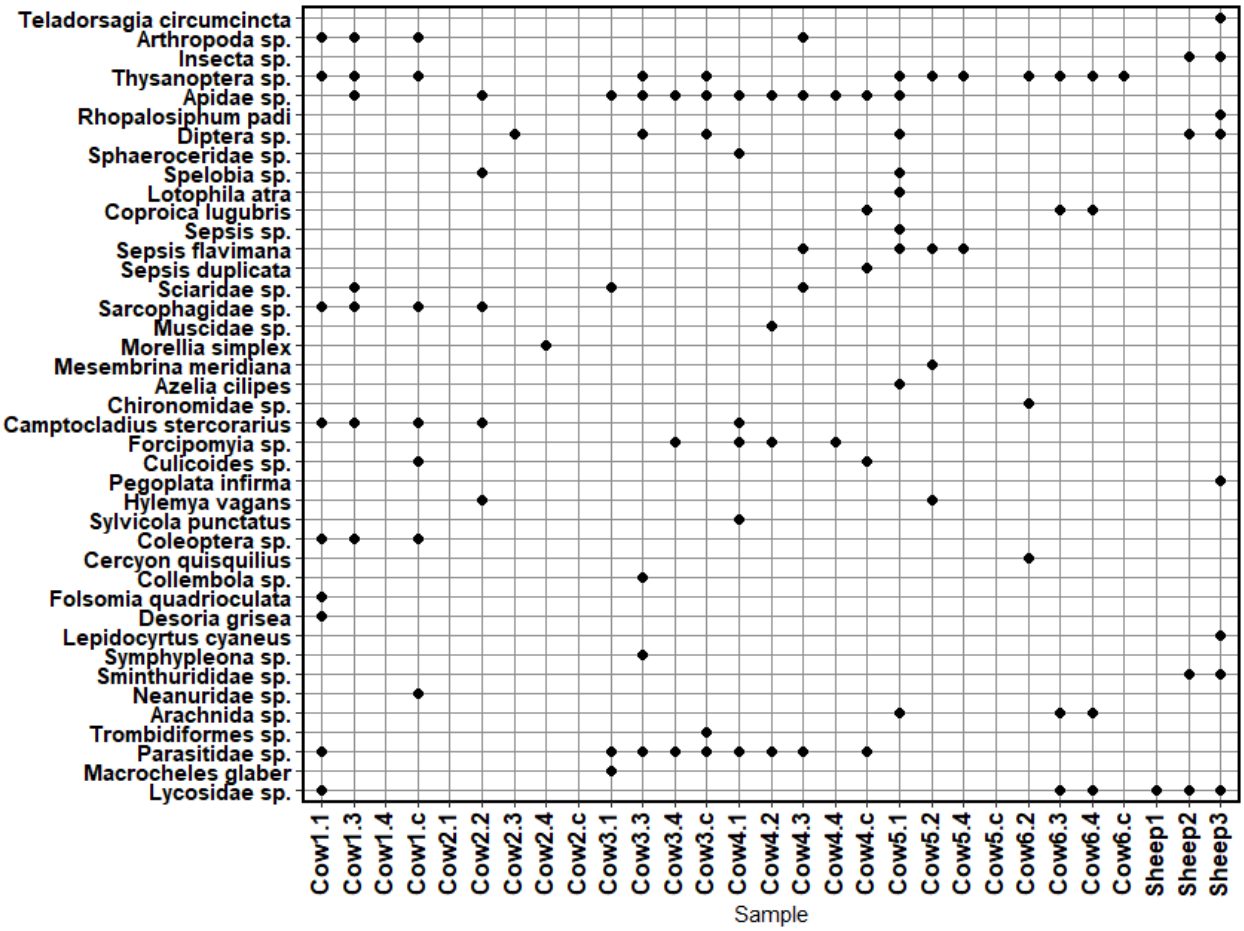

Figure 7: The proportion of the target sequencing reads allocated to the different taxa (rows) within each sample (columns) where they were detected using the BF3/BR2 assay. Each bubble per sample represents the proportion of DNA for a target species in that sample. The size of the bubble is relative to the number of sequences from all species for that sample. The colour of the bubble represents the taxonomic class.

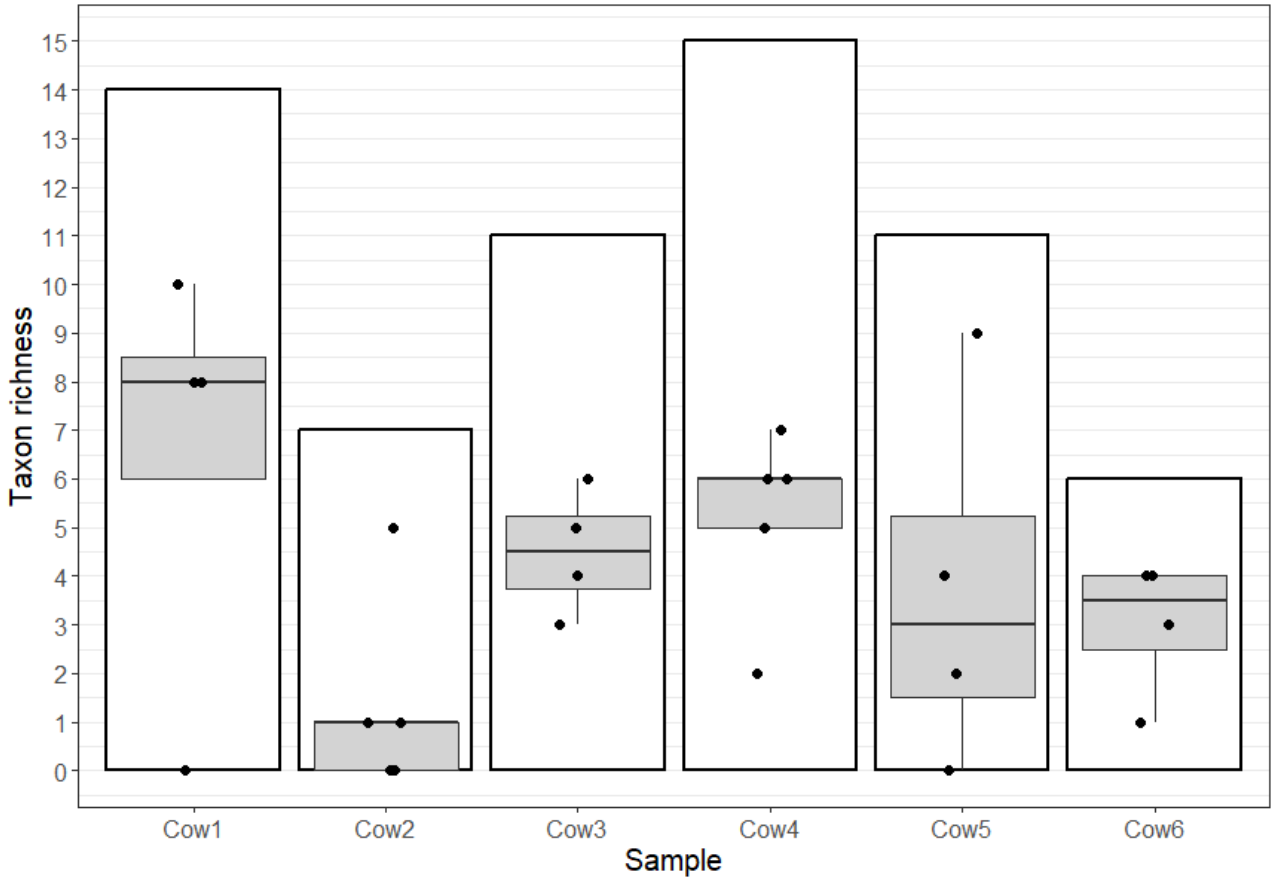

Figure 8: Taxon richness (number of OTUs) for invertebrate communities within each cowpat detected using the BF3/BR2 assay. The bar shows cowpat-level taxon richness (cumulative richness for each cowpat). The boxplot shows subsample-level richness, with the box depicting the median between the upper/lower quantiles, the whiskers indicating minimum and maximum values, and dots showing richness values of each sample. Any samples beyond the whiskers are considered as outliers which are 1.5x the interquartile-range away from the upper or lower quartile.

mlCOIintF_XT/jgHCO2198 Assay

The mlCOIintF_XT/jgHCO2198 assay detected 82 target OTUs belonging to five phyla (Arthropoda, Annelida, Mollusca, Nematoda and Rotifera) across all the samples. The minimum species richness was 4 (Cow2.1, Cow2.C, Cow5.3, Cow6.1). The maximum species richness was 15 (Cow5.1). The OTU with the highest proportion of reads across samples was from the thrip order Thysanoptera and was detected in 16/33 samples. One OTU from the order Hemiptera was detected in all samples.

Of the OTUs that were identified to species level, the taxa with the greatest proportion of sequencing reads across the samples were a species of root-maggot fly (*Hylemya vagans*), a species of slender springtail (*Lepidocyrtus cyaneus*), a Sepsidae fly (*Sepsis flavimana*), and the Hedgehog slug (*Arion intermedius*), and the taxa with the highest frequency of detections were a non-biting midge (*Camptocladius stercorarius,* present in

seven samples), a Sepsidae fly (*Sepsis flavimana,* five samples) and an intestinal parasitic nematode (*Cooperia onchophora*, five samples).

Species of note detected by the assay include *Cooperia onchophora and Ostertagia ostertagi*, both parasitic nematodes in cattle.

Some differences in invertebrate community composition were observed among samples (Figure 9). The replicates from Cow 2 were more variable then those within other faecal deposits, with the 95% confidence interval overlapping that of all but one other Dung IDs (Cow 4). Cow 6 had the least variation between replicates with the points clustering closely together. Cow 5 and Cow 6 show most similarity with one another, as do Cow 3 and Cow 4. The invertebrate community compositions of all the sheep samples are more closely related to one another than that of the majority of cow samples (exceptions in relation to Cow 2).

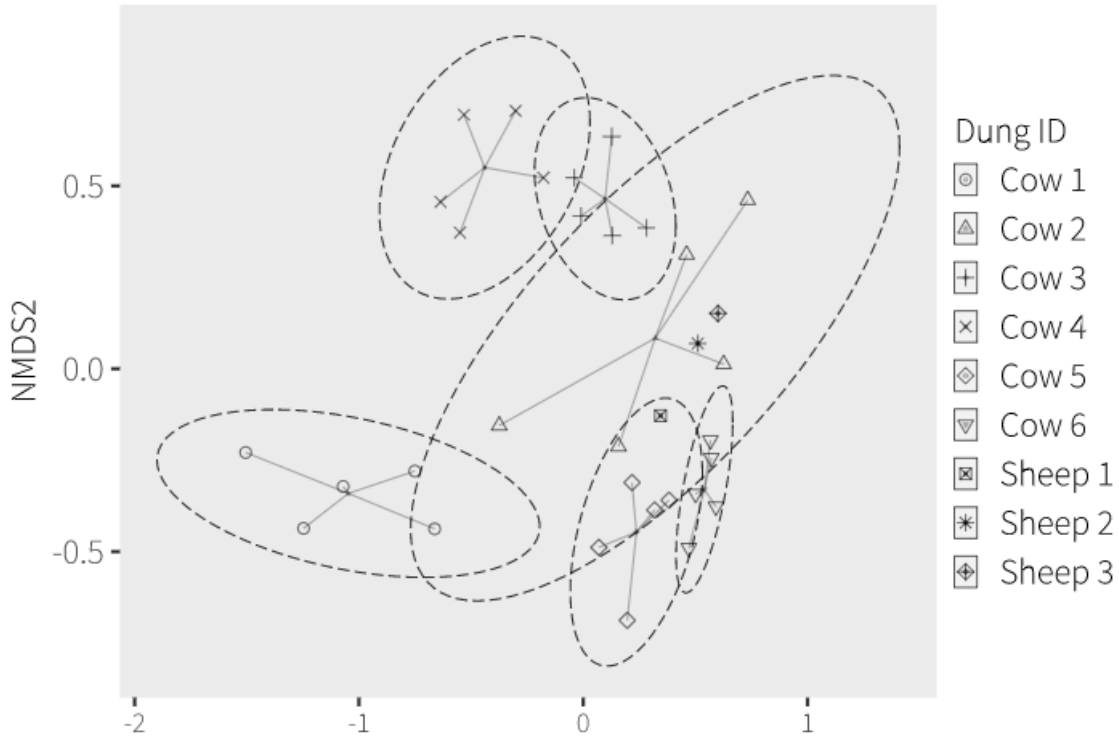

Figure 9: NMDS ordination plot based on Jaccard similarity index for target taxa in cow faeces using the mlCOIintF_XT/jgHCO2198 assay. Point shape denotes stand Dung ID. 95% confidence intervals for each faecal deposit are indicated by dashed ellipses. Replicate samples collected from the same faecal deposit are connected with a solid line.

A bubble plot of the assay's target detections across all successful samples is provided in Figure 10. Cow samples are dominated by class Insecta. For the cow originating samples, Coleoptera sp. (Cow 1), Hemiptera sp. (Cow 2 and Cow 3), Apidae sp. (Cow 4) and Thysanoptera (Cow 5 and Cow 6), characterise samples. Additionally, Hemiptera sp. occurs in all samples and represent the greatest percentage of reads in all of the sheep samples.

Cow 2 and 5 faecal deposits had the highest cumulative taxon richness and Cow 5 had the highest median richness per replicate (Figure 11).

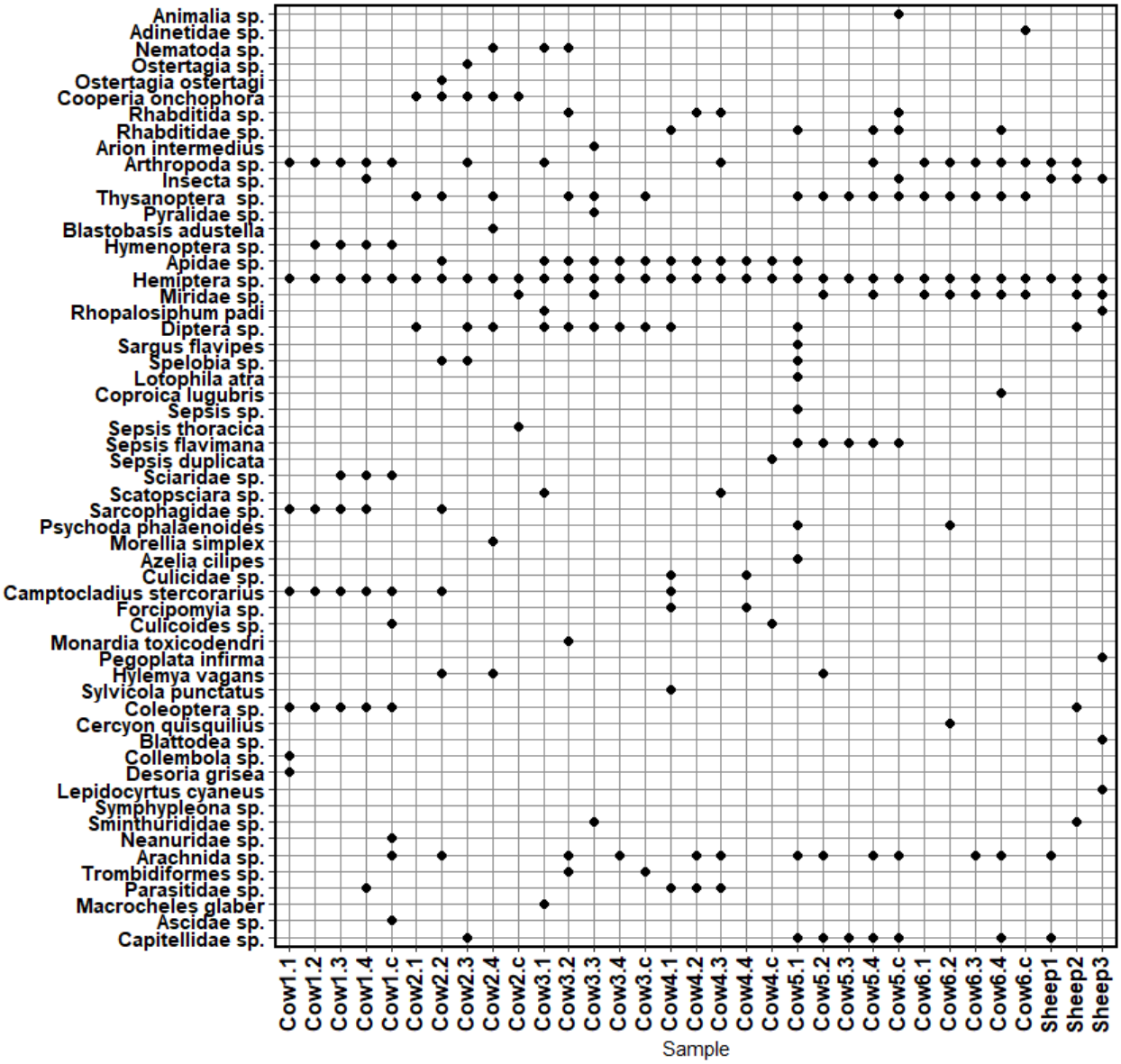

Figure 10: The proportion of the sequencing output allocated to the different species (rows) within each sample (columns) where they were detected using the mlCOIintF_XT/ jgHCO2198 assay. Each bubble per sample represents the proportion of DNA for a target species in that sample. The size of the bubble is relative to the number of sequences from all species for that sample. The colour of the bubble represents the taxonomic class.

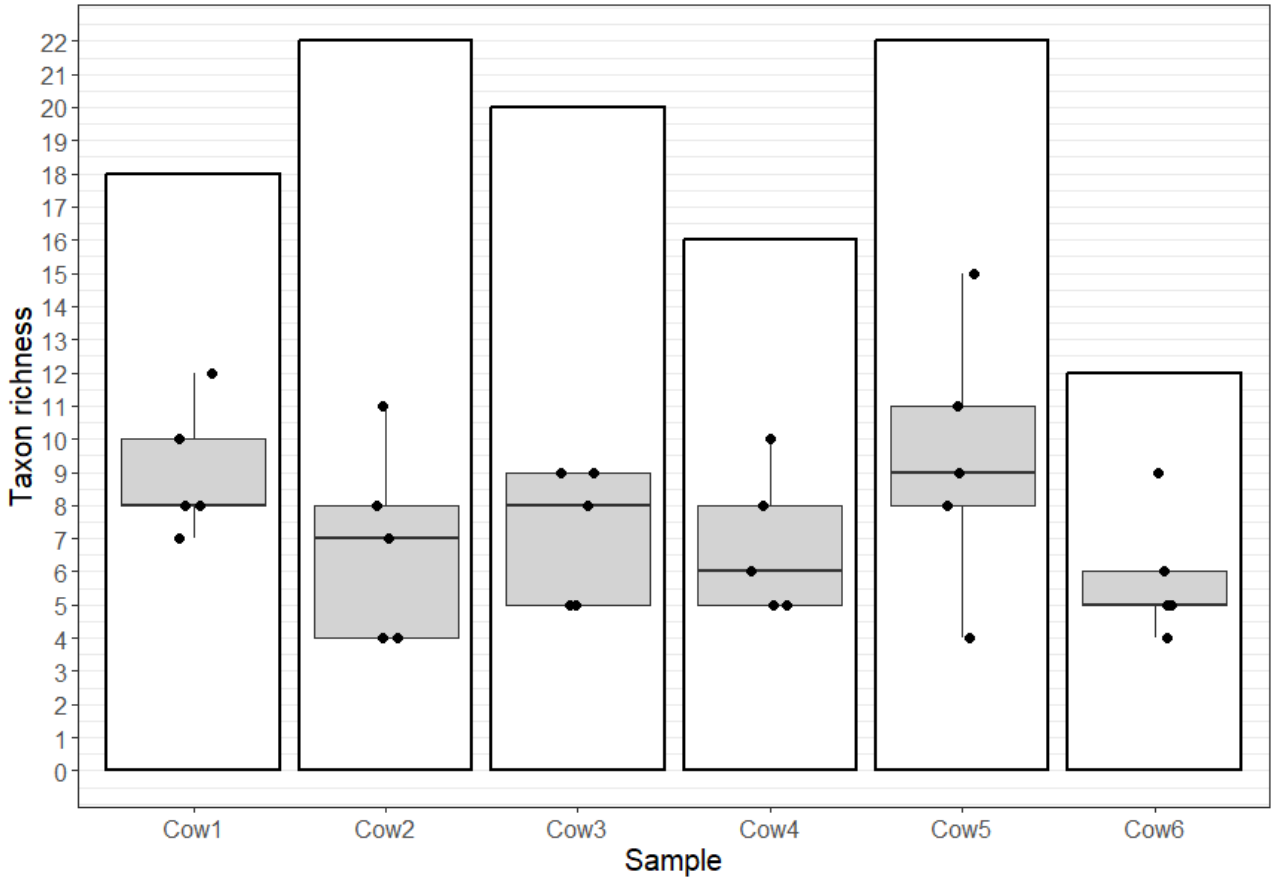

Figure 11: Taxon richness (number of OTUs) for invertebrate communities within each cowpat detected using the mlCOIintF_XT/jgHCO2198 assay. The bar shows cowpat-level taxon richness (cumulative richness for each cowpat). The boxplot shows subsample-level richness, with the box depicting the median between the upper/lower quantiles, the whiskers indicating minimum and maximum values, and dots showing richness values of each sample. Any samples beyond the whiskers are considered as outliers which are 1.5x the interquartile-range away from the upper or lower quartile.

Assay comparison

A summary of the sequence read outcomes for each assay is provided in Table 5. The mlCOIintF_XT/jgHCO2198 assay generated the greatest number of total reads and the greatest number of target reads, followed by the BF3/BR2 assay and the BF2/BR2 assay. The percentage of reads that belonged to target taxa was only 4.3% for the mlCOIintF_XT/jgHCO2198 assay, 3.5% for the BF2/BR2 assay, and 2.9% for the BF3/BR2 assay.

The total number of target taxa detected across samples (Table 6) was greatest for the mlCOIintF_XT/jgHCO2198 assay (82) followed by BF3/BR2 (55) and BF2/BR2 (34). The mlCOIintF_XT/jgHCO2198 assay detected the most phyla across samples (Arthropoda, Annelida, Mollusca, Nematoda and Rotifera) compared to the BF3/BR2 (Arthropoda and Nematoda) and BF2/BR2 assay (Arthropoda). Mean richness across samples and

maximum richness of samples was highest for the mlCOIintF_XT/jgHCO2198 assay followed by the BF3/BR2 assay and BF2/BR2 assay. The BF3/BR2 assay identified the greatest proportion of detected target OTUs to both genus and species level followed by mlCOIintF_XT/ jgHCO2198 and BF2/BR2 (Table 7).

Table 5: Summary of sequence read outcomes per assay

Primers	Total Reads	Total Target Reads (as % of total reads)	Total Non-Target Reads	Total unassigned reads
BF2/BR2	1 035 387	383 039 (36.99%)	9 971	615 616
BF3/BR2	1 468 825	567 166 (38.61%)	25 045	876 614
mlCOIintF_XT/ jgHCO2198	1 629 617	714 454 (43.84%)	89 027	826 136

Table 6: Summary of target taxa richness for all samples for each assay

Primers	Total Target Taxa	No. of Phyla	Mean Richness	Min Richness	Max Richness
BF2/BR2	34	1	3.4	1	7
BF3/BR2	55	2	4.92	1	12
mlCOIintF_XT/ jgHCO2198	82	5	7.55	4	15

Table 7: Percentage of OTUs assigned to each taxonomic rank across samples for each assay

Primers	Phylum	Class	Order	Family	Genus	Species
BF2/BR2	100%	97.2%	76.5%	64.7%	29.4%	26.5%
BF3/BR2	100%	98.2%	80%	69.1%	40%	32.7%
mlCOIintF_XT/ jgHCO2198	98.8%	93.9%	75.6%	56.1%	35.4%	28%

Table 8 shows a summary of detections at species level across the three assays targeting invertebrate species. The mlCOIintF_XT/jgHCO2198 assay recovered the greatest assignments at species level, followed by BF3/BR3, and finally BF2/BR2. Of the 26 species, 8 were found in all three assays. The coincidence and singularity of OTU detections in relation to combinations of the three assays are summarised in a Venn

diagram (Figure 12). The assay returning the greatest number of unique assignments is mlCOIintF_XT/jgHCO2198, greater than those sharded by all assays. This is followed by assignments shared by mlCOIintF_XT/jgHCO2198 and BF3/BR2, and BF3/BR2 alone.

Table 8: Species detection by assay. Some cells have been intentionally left blank.

Phylum	Class	Order	Species	BF2/ BR2	BF3/ BR2	mlCOIintF_XT/ jgHCO2198
Arthropoda	Arachnida	Mesostigmata	*Macrocheles glaber*		x	x
Arthropoda	Collembola		*Lepidocyrtus cyaneus*	x	x	x
Arthropoda	Collembola		*Desoria grisea*	x	x	x
Arthropoda	Collembola		*Folsomia quadrioculata*	x	x	
Arthropoda	Insecta	Coleoptera	*Cercyon quisquilius*	x	x	x
Arthropoda	Insecta	Diptera	*Sylvicola punctatus*		x	x
Arthropoda	Insecta	Diptera	*Hylemya vagans*	x	x	x
Arthropoda	Insecta	Diptera	*Pegoplata infirma*	x	x	x
Arthropoda	Insecta	Diptera	*Monardia toxicodendri*			x
Arthropoda	Insecta	Diptera	*Camptocladius stercorarius*	x	x	x
Arthropoda	Insecta	Diptera	*Azelia cilipes*		x	x
Arthropoda	Insecta	Diptera	*Mesembrina meridiana*		x	
Arthropoda	Insecta	Diptera	*Morellia simplex*	x	x	x
Arthropoda	Insecta	Diptera	*Psychoda phalaenoides*			x
Arthropoda	Insecta	Diptera	*Sepsis duplicata*		x	x
Arthropoda	Insecta	Diptera	*Sepsis flavimana*	x	x	x
Arthropoda	Insecta	Diptera	*Sepsis thoracica*			x
Arthropoda	Insecta	Diptera	*Coproica lugubris*		x	x
Arthropoda	Insecta	Diptera	*Lotophila atra*		x	x
Arthropoda	Insecta	Diptera	*Sargus flavipes*			x
Arthropoda	Insecta	Hemiptera	*Rhopalosiphum padi*		x	x
Arthropoda	Insecta	Lepidoptera	*Blastobasis adustella*			x
Mollusca	Gastropoda	Stylommatophora	*Arion intermedius*			x
Nematoda	Secernentea	Strongylida	*Cooperia onchophora*			x
Nematoda	Secernentea	Strongylida	*Ostertagia ostertagi*			x
Nematoda	Secernentea	Strongylida	*Teladorsagia circumcincta*		x	

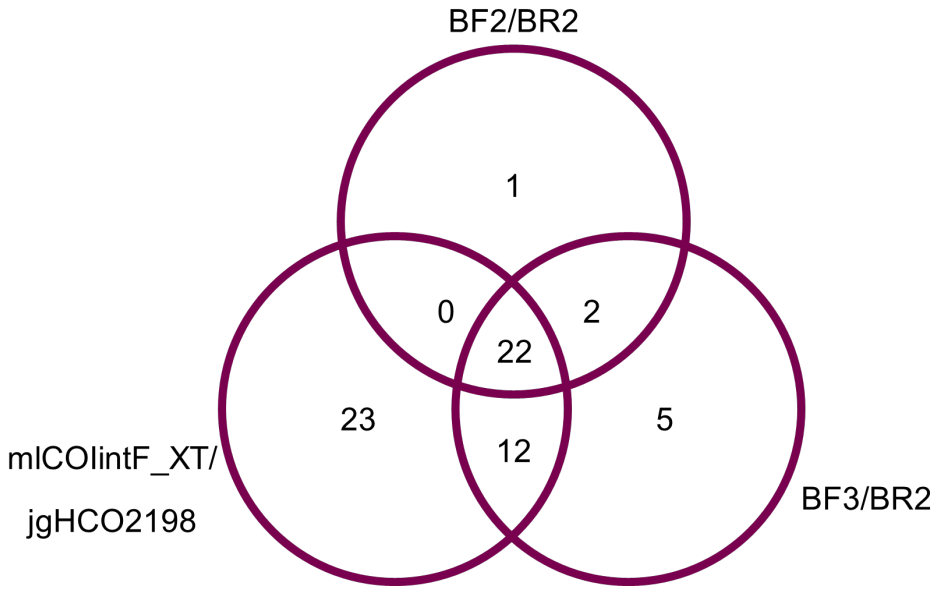

Figure 12: Venn diagram summarising taxa detections per assay, taxa detected with multiple assays, and taxa detected only by individual assays

There were some similarities in the taxa detected across the assays, but with much greater breadth of diversity captured with mlCOIintF_XT/jgHCO2198 compared to the other assays. The number of assignments to family (or below level) increased from BF2/BR2, to BF3/BR2 and to mlCOIintF_XT/jgHCO2198. The absence of Nematoda is noticeable from the BF2/BR2 assay, as is the substantially increased occurred in this taxon in mlCOIintF_XT/jgHCO2198. The greatest richness in taxa at family level sit within Diptera for all assays.

Evaluation and recommendations

This pilot demonstrates that eDNA collected from cow and sheep faeces does detect signatures from terrestrial invertebrates and may be a valuable source to investigate community compositions and associations. Both taxonomic and functional diversity are represented across the eDNA detections. Of the 23 Arthropod taxa identified to species level, only two had no known association with livestock faeces. 16 of the species detected have a direct reliance on faeces, either as food sources, e.g., *Cercyon quisquilius*, or for breeding, e.g. *Sepsis flavimana*. Other dung-associated invertebrate functional groups detected include fungal feeders (e.g., springtails, mites) and parasites (e.g., nematodes).

As an approach for monitoring of dung beetles, the application of this method appears limited. Only two dung beetle families occur in the UK, Geotrupidae and Scarabaeidae, neither of which were detected across any of the samples or assays. Though the Coleoptera order was detected during sampling, the single assignment to species was of the Hydrophilidae family, *Cercyon quisquilius*. Although dung beetles are known to occur in the area, and tunnels were observed in the faeces during sampling, no specimens were visually observed. We therefore cannot confirm whether dung beetles should have been

detected in this study or not. Dung beetles have previously been detected from faecal eDNA. In a study by Sigsgaard and others., 2021, four species belonging to the Scarabaeidae family were detected from samples collected across nine cowpats, using a different COI primer set.

It is also interesting to note the presence of three parasitic nematodes in the dataset: two for cattle and one for sheep, particularly in light of the cessation of routine worm treatment on the farm estate since 2018/2019, and the lack of stock worming in 2020. Their respective occurrences were restricted to the appropriate livestock species, with the cattle parasites being recorded using the mlCOIintF_XT/jgHCO2198 assay, and sheep parasites with the BF3/BR2 assay. The faecal deposit with the greatest occurrence of parasites, and the only one in which *Ostertagia ostertagi* was recorded, was Cow 2. In general, the results from this faecal deposit, partly due to reduced sample diversity, and part the taxonomic differences to other samples and replicates, were unable to be displayed on some of the included figures. The ability of the assays to determine parasitic nematodes to species level alongside other invertebrate, may be a coincidental benefit of the eDNA techniques, complimenting Faecal Egg Count data.

The eDNA approach taken here has the potential to be developed into a standard monitoring method by which to assess wider dung-associated invertebrate communities. Further research would be required to determine its efficacy in specific relation to dung beetles, and consequently the ability to use members of the taxa as indicators of good soil management and any consequent applications in regenerative farming.

Sampling kit and method

The success in amplifications, and taxon recovery across all assays demonstrates the ability of this sampling method to generate invertebrate community data from faecal samples. Though the per sample sequencing depth of target taxa was low, read numbers per invertebrate taxa are comparable to those in previous work (Sigsgaard and others., 2021). While sampling was conducted by NatureMetrics staff for this study, the sampling kit and protocol used to collect samples does not require specialised knowledge or training to use. The same method could be rolled out for a citizen science project to monitor invertebrate communities associated with faecal samples.

It is logistically challenging to collect whole cowpat samples for DNA analysis. A subsampling method is therefore required. Detection success can vary among species in faecal DNA (Thuo and others., 2019) and detections are often distinct among faecal sampling locations within a cowpat (Gosselin and others., 2017). This was also demonstrated in this project with variation in community composition observed among replicates from the same faecal deposit. This variation suggests that subsampling is an essential method to improve invertebrate assemblage information gathered via eDNA techniques, in this case for faecal DNA.

Homogenised faecal samples have greater DNA detection rates when compared to single subsamples from individual locations (Deagle and others., 2005). The data presented in this report for cow faeces are not homogenisations of replicates. This was a conscious decision to retain between subsample variance information, and to subsequently inform any future sampling strategy. However, there is potential to combine the subsamples into a composite samples, as was done in this study with the sheep samples, to get a more representative sample for each faecal deposit while reducing analysis costs. No investigation of the optimal number of subsamples was made here. A balance between sampling intensity and sampling efficiency was made decided on prior to sampling, in accordance with methods published previously (Sigsgaard and others., 2021).

Each sample comprises of subsamples to account for the potential of localised distribution of DNA within whole faecal deposits. Sample replicates from the site are required to account for the potential of localised distribution of invertebrates between individual faecal deposits. Although a distinction is less clear in mlCOIintF_XT/jgHCO2198, Cow 1 and its subsamples appear to be less similar to other samples and subsamples. With the exception of Cow 1, all samples were taken from a lowland pasture area of the farmland. Since invertebrate communities differ between habitats, there would be an expectation for this to be mirrored in the faecal community. In-dung taxa assemblages have been previously shown to differ between habitats (Sigsgaard and others., 2021), however, only a single sample was taken from the heathland area for this project, and as such no conclusive comparisons can be made.

Assay choice

When assessing the most efficient way to analyses invertebrate eDNA in faeces as part of this project, there are two key factors to consider: the breadth of the assays versus the resolution and the ability to detect target species. The original focus of the project was on dung beetles, Geotrupidae and Scarabaeidae. Considering this, no assay detected these taxa within these samples. Widening to other invertebrate taxa, if the intent is to get high-resolution, species-level data, then the BF3/BR2 assay had slightly higher species- and genus-level assignments than the other two. However, this assay failed to amplify in four samples, the only assay to do so, and a further four did not return any target taxa. BF3/BR2 is less affected by primer slippage than BF2/BR2 and therefore provides maximal taxonomic resolution (Elbrecht and others., 2019). Although the species detection rate was lower for mlCOIintF_XT/jgHCO2198 in comparison to BF3/BR2, the number of both genus and species detected and taxon richness across all samples was higher.

For broad range detections across multiple taxonomic classes, the mlCOIintF_XT/jgHCO2198 assay can be recommended. The mlCOIintF_XT/jgHCO2198 assay outperforms the other assays in terms of taxa detected (albeit with slightly lower taxonomic resolution) but is more robust against the wider sample set derived from the faeces pilot project. The mlCOIintF_XT/jgHCO2198 primer set amplifies a shorter gene fragment which is ideal when targeting degraded DNA (e.g., from faeces). The use of

BF2/BR2 for terrestrial invertebrate monitoring in faeces would not be recommended based on the results obtained in this project.

The importance of dung beetle species as indicators to determine the health of soils in an agricultural management context needs to be considered when appraising available and new assay options in the future. Further work would be required to determine the effectiveness of invertebrate or wider metazoan targeting assays in relation to detection of dung beetles. More specific primers may be required to target Coleoptera, or dung beetles specifically, if the project aims were strictly related to these taxa.

As a pilot project involving only six individual cowpats and three sheep faecal samples, collected from a single geographic site at one sampling time point, there is limited environmental-covariate data. It is therefore not possible to derive robust insights for soil management assessment. Instead, the value of this project is in the information it has provided on the ability of eDNA techniques to detect invertebrates across taxonomic breadth from faecal samples, the sampling method, and in the taxa that are detected using different COI primer sets, all of which would be necessary for any scaling up in the context of a potential citizen science project.

Faecal eDNA metabarcoding has the promise to efficiently provide invertebrate community data, to a high-level, across large scale areas, to a standardised degree. With repeated sampling, faecal eDNA metabarcoding in agricultural environments can be used to track changes in species occupancies, and if combined with relevant taxa ecological and functional information, could inform implications on soil and to an extent, wider ecosystem health.

Limitations

Assigning taxonomic identities to the sequences is only possible through their comparison to reference databases, which are incomplete. This is not an issue if a taxonomy free approach is adopted – i.e. tracking changes over time by comparing datasets (as is advocated here), but it is a bigger concern if indicator species or functional groups (based on taxonomy) are required. It should be noted that multiple OTUs can be identified as belonging to the same species, which is most likely attributed to PCR or sequencing artefacts but potentially intraspecific genomic variation or cryptic diversity. Also, it is possible for closely related species to have identical sequences in the targeted gene region and if the species present at your site is not in the database it could be identified as a different closely related species.

The abundance of taxa cannot be directly inferred from the number of sequence reads. While the number of sequence reads is a consequence of abundance, it is also impacted by biomass, body type, activity, surface area, condition, primer bias, and species-specific variation in the genome. However, a higher proportion of a taxon in the assay can be interpreted that the species detection is more confident in the respective sample.

References

Buse, J., Šlachta, M., Sladecek, F. X. J., Pung, M., Wagner, T., and Entling, M. H. 2015. Relative importance of pasture size and grazing continuity for the long-term conservation of European dung beetles. Biological Conservation, 187:112–119. DOI: 10.1016/j.biocon.2015.04.011

Deagle, B.E., Tollit, D.J., Jarman, S.N., Hindell, M.A., Trites, A.W. and Gales, N.J., 2005. Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. Molecular Ecology, 14(6):1831-1842.

Elbrecht, V. and Leese, F., 2017. Validation and development of COI metabarcoding primers for freshwater macroinvertebrate bioassessment. Frontiers in Environmental Science, 5: 11. DOI: 10.3389/fenvs.2017.00011

Elbrecht, V., Braukmann, T.W.A., Ivanova, N.V., Prosser, S.W.J., Hajibabaei, M., Wright, M., Zakharov, E.V., Hebert, P.D.N. and Steinke, D., 2019. Validation of COI metabarcoding primers for terrestrial arthropods. PeerJ, 7: e7745. DOI: 10.7717/peerj.7745.

Filgueiras, B.K., Tabarelli, M., Leal, I.R., Vaz-De-Mello, F.Z. and Iannuzzi, L., 2015. Dung beetle persistence in human-modified landscapes: combining indicator species with anthropogenic land use and fragmentation-related effects. Ecological Indicators, 55: 65-73.

Gosselin, E.N., Lonsinger, R.C. and Waits, L.P., 2017. Comparing morphological and molecular diet analyses and fecal DNA sampling protocols for a terrestrial carnivore. Wildlife Society Bulletin, 41(2): 362-369.

Leray, M., Yang, J.Y., Meyer, C.P., Mills, S.C., Agudela, N., Ranwez, V., Boehm, J.T. and Machida, R.J., 2013. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. Frontiers in Zoology, 10: 34. DOI: 10.1186/1742-9994-10-34

Skidmore, P., 1991. Insects of the British cow-dung community. Field Studies Council.

Thuo, D., Furlan, E., Broekhuis, F., Kamau, J., MacDonald, K. and Gleeson, D.M., 2019. Food from faeces: Evaluating the efficacy of scat DNA metabarcoding in dietary analyses. PLoS One, 14(12): p.e0225805.

Tonelli, M., Verdú, J. R., and Zunino, M. 2018. Effects of the progressive abandonment of grazing on dung beetle biodiversity: Body size matters. Biodiversity and Conservation, 27(1): 189–204. DOI: 10.1007/s10531-017-1428-3

Wangensteen, O.S., Palacin, C., Guardiola, M. and Turon, X., 2018. DNA metabarcoding of littoral hard-bottom communities: high diversity and database gaps revealed by two molecular markers. PeerJ, 6: e4705; DOI: 10.7717/peerj.4705

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Appendices

Appendix A

Table 1 Photographs of each sample taken by Kate Denton.

Sample ID	Photo of sample
Cow1	
Cow2	
Cow3	

Sample ID	Photo of sample
Cow4	
Cow5	
Cow6	
Sheep1	

Sample ID	Photo of sample
Sheep2	
Sheep3	

Appendix B

Table 2 Faecal sample and sample replicate identifications with associated assays and whether they proceeded or dropped out of the analysis based on the following categories: 1 - Samples with target taxa reported; 2 - Samples which failed troubleshooting prior to sequencing; 3 - Samples with only environmental contaminant sequences.

Sample ID	Originator animal	BF2/BR2	BR3/BR2	mlCOIintF_XT/ jgHCO2198
Cow1.1	Cow	1	1	1
Cow1.2	Cow	1	2	1
Cow1.3	Cow	1	1	1
Cow1.4	Cow	1	3	1
Cow1.C	Cow	1	1	1
Cow2.1	Cow	3	3	1
Cow2.2	Cow	1	1	1
Cow2.3	Cow	3	1	1
Cow2.4	Cow	1	1	1
Cow2.C	Cow	3	3	1
Cow3.1	Cow	1	1	1
Cow3.2	Cow	1	2	1
Cow3.3	Cow	1	1	1
Cow3.4	Cow	1	1	1
Cow3.C	Cow	1	1	1

Sample ID	Originator animal	BF2/BR2	BR3/BR2	mlCOIintF_XT/ jgHCO2198
Cow4.1	Cow	1	1	1
Cow4.2	Cow	1	1	1
Cow4.3	Cow	1	1	1
Cow4.4	Cow	1	1	1
Cow4.C	Cow	1	1	1
Cow5.1	Cow	1	1	1
Cow5.2	Cow	1	1	1
Cow5.3	Cow	1	2	1
Cow5.4	Cow	1	1	1
Cow5.C	Cow	1	3	1
Cow6.1	Cow	1	2	1
Cow6.2	Cow	1	1	1
Cow6.3	Cow	1	1	1
Cow6.4	Cow	1	1	1
Cow6.C	Cow	1	1	1
S1	Sheep	1	1	1
S2	Sheep	1	1	1
S3	Sheep	1	1	1

www.gov.uk/natural-england