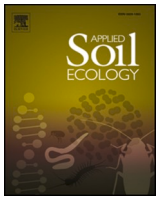
Applied Soil Ecology 222 (2026) 106996

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil



Research paper

Environmental RNA and DNA metabarcoding of soil fauna reveal complementary insights into biodiversity and limited effect of nitrification inhibitors

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ARTICLE INFO

Keywords:
Arthropods
High throughput sequencing
Soil health
Collembola
Earthworms
COI

ABSTRACT

Metabarcoding of environmental DNA (eDNA) is rapidly being adopted to describe and assess biodiversity. However, eDNA has limitations since it captures DNA from both living and dead organisms including relic and extracellular DNA and cannot distinguish metabolically active individuals. In contrast, environmental RNA (eRNA) has the potential to reflect the living organisms. In this study, we compared eRNA and eDNA metabarcoding analysis of soil fauna communities from a cultivated field plot experiment across two seasons to assess the active taxa (eRNA) to the total taxa (eDNA) and hence soil fauna diversity. Both approaches showed distinct community structures, including a large overlap in OTUs shared between the two methods. Seasonal effects were evident in both methods; eDNA detected a higher number of species than eRNA in spring; however, the opposite was observed in fall. Soil fertilizer effect was detected in both approaches, while the effects of nitrification inhibitors (NI) concentration were only significant in the eRNA spring dataset shortly after NI and fertilizer application. This indicates that while eDNA reflects the overall soil fauna diversity, eRNA provides a more sensitive snapshot of the living, transcriptionally active fraction of the community. Our results highlight that eDNA and eRNA metabarcoding of soil fauna provide different but complementary insights into soil biodiversity, creating new opportunities in monitoring soil health and ecosystem functions in soil ecological studies.

1. Introduction

Soils are among the most biologically diverse habitats on Earth, harboring a diverse variety of organisms, from microbes to invertebrates, that contribute significantly to ecosystem processes (Delgado-Baquerizo et al., 2020). Soil biodiversity forms the foundation of terrestrial ecosystems and is key to sustaining a wide range of ecosystem services, including organic matter decomposition, nutrient cycling, soil structure formation, and plant productivity (Wagg et al., 2014). Among soil biota, micro-, meso-, and macrofauna, – such as springtails, earthworms, nematodes, and mites, – are among the most diverse and functionally important (Coleman and Whitman, 2005). These soil fauna taxa interact with soil microbes and contribute to key soil biological processes, including soil health (Lavelle et al., 1997). However, despite their critical roles in soil food webs and ecosystem functioning, soil fauna remains underrepresented in studies of terrestrial ecosystems, particularly concerning the seasonal dynamics and response to anthropogenic activities such as land use and agricultural intensification.

Anthropogenic activities often impact the community structure of soil organisms. Agricultural practices such as tillage, chemical fertilizer application, and crop cultivation collectively form crop management strategies that significantly disturb soil ecosystems. Nitrogen is a major element supplemented to crop production as chemical fertilizer or organic manure, primarily in the form of ammonium and nitrate. However, these forms of nitrogen are susceptible to losses in soil through leaching to aquatic recipients, conversion to nitrates and nitrous oxide, or gaseous emissions into the atmosphere (Cameron et al., 2013;

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https://doi.org/10.1016/j.apsoil.2026.106996
Received 30 July 2025; Received in revised form 28 January 2026; Accepted 26 March 2026
Available online 31 March 2026

Jackson et al., 2008; Petersen, 1999). Recently developed nitrification inhibitors (NI) inhibit ammonium conversion to nitrates, thus allowing longer availability of ammonium to crops (Kim et al., 2012; Peixoto and Petersen, 2023). However, the effects of these NI on non-target soil organisms, especially soil fauna, are largely unknown. Traditional morphological identification methods to evaluate soil fauna often become challenging and require extensive taxonomic expertise of a wide range of species (Sapkota and Nicolaisen, 2015).

To address these limitations, recent progress in molecular methodologies, as environmental DNA (eDNA) metabarcoding, has been increasingly used for the assessment of soil biodiversity (Jänsch et al., 2025; Sapkota et al., 2025; Taberlet et al., 2018). Metabarcoding of eDNA allows the identification of a broad range of species through their extracellular DNA fragments they leave in their surroundings and their intracellular DNA from intact organisms. Compared to traditional surveys, it is less invasive, requires smaller soil volumes, causes less disturbance to the experimental plots, and captures a large range of biodiversity (Lilja et al., 2023; Sapkota and Nicolaisen, 2018).

Environmental RNA (eRNA) is increasingly being applied in ecological surveys of both eukaryotes and prokaryotes across a wide range of environments, including marine sediments (Guardiola et al., 2016), freshwater (Miyata et al., 2022), forest soil (Romanowicz et al., 2016), ballast water (Xue et al., 2024), and soil environments (Adamo et al., 2020). In these studies, environmental RNA metabarcoding follows a common methodological framework based on immediate sample preservation, DNase treatment to remove residual DNA, reverse transcription, and amplification of barcode targets. RNA quality is therefore defined operationally by the ability to generate reproducible barcode amplicons rather than by transcriptomics-oriented integrity metrics such as RIN values or electropherogram profiles.

The eRNA metabarcoding performs like the eDNA metabarcoding and might overcome some of its limitations (Yates et al., 2021). Unlike eDNA, which has been reported to persist in the environment for a long time (Carini et al., 2016), eRNA is more transient and degrades rapidly in the environment, making it a reliable indicator of active organisms. Hence, eRNA offers a more dynamic perspective of metabolically active taxa, whereas eDNA provides a wide overview of living and extinct organisms, including those that are inactive, and those that are represented by remains or deceased (Kagzi et al., 2023; Pochon et al., 2017). eRNA metabarcoding is one step further in detecting active organisms in a given environment and is hypothesized to provide a more sensitive picture of biodiversity across time and space (Adamo et al., 2020). While studies using eRNA in soil ecosystems are still relatively rare, studies from aquatic ecosystems have shown that it can serve as a powerful tool to track changes in biodiversity and portray a more dynamic view of community stability (Ankley et al., 2022; Veilleux et al., 2021).

Although bioinformatics techniques and algorithms remove some artifacts of artifactual sequences generated during PCR amplification or sequencing, each method still comes with its limitations (Prodan et al., 2020; Rognes et al., 2016). It has been suggested that analyzing OTUs shared between eDNA and eRNA datasets might be a future approach to increase reliability. Integrating eDNA and eRNA, collectively, also referred to as environmental nucleic acids (eNAs), in soil fauna metabarcoding research can overcome some of the shortcomings of eDNA metabarcoding and thus enable a more comprehensive study of ecological processes and biodiversity (Veilleux et al., 2021).

In soil fauna biodiversity studies, the cytochrome *c* oxidase subunit I (COI) gene is the most widely used DNA barcode for species identification and community assessments of metazoans (Andújar et al., 2018; Hebert et al., 2003). As part of the mitochondrial genome, COI is a protein-coding gene, and, once transcribed into messenger RNA (mRNA), present in the eRNA pool extracted from soil. However, eRNA mainly includes ribosomal RNA (rRNA), with mRNA often only present as a small fraction of the soil eRNA (Bang-Andreasen et al., 2020). In contrast to rRNA, which includes relatively stable molecules such as 18S and 28S rRNA that accumulate in the cytoplasm, mRNA has a shorter half-life in soil, making it a potential marker of recent transcriptional activity (Kunadiya et al., 2021). Hence, eRNA-based metabarcoding of COI should provide a dynamic picture of active soil fauna communities, distinguishing metabolically active organisms from dormant or dead ones that contribute to the eDNA pool.

Our objectives were to evaluate the effectiveness of eRNA and eDNA metabarcoding in identifying key drivers of soil fauna in cultivated soil, and to investigate the response of soil fauna communities to fertilizer and NI applications. We assessed the potential of eRNA as a proxy for active soil fauna diversity from total soil fauna diversity by eDNA by comparing the seasonal community profiles using the COI marker gene-based metabarcoding. We hypothesized that a) eRNA outperforms eDNA in short term ecological response by capturing the active soil community, b) seasonal variation in soil fauna is distinct by eRNA compared to eDNA, c) the application of NI affects the active community composition, and such effect is readily detected in the eRNA datasets. Using eDNA and eRNA of the mitochondrial gene COI, we tested the impact of inorganic and organic fertilizers, and of two NIs at three concentrations on the soil fauna community across two seasons in Danish cultivated soil. By sampling the same field plots across two seasons, we compared the species richness and community structure, derived from each nucleic acid type. With this work, we contribute to integrating RNA-based approaches into soil ecology and activity-based monitoring of soil invertebrate communities.

2. Materials and methods

2.1. Experimental setup and soil sampling

Soil samples were collected from a long-term field trial located at Højbakkegård, Taastrup, Zealand (55.67158 N, 12.300222 E), Denmark with winter wheat designed as a randomized complete blocks experiment with 3 replicates (blocks) as described earlier (Lilja et al., 2023; Sapkota et al., 2025; Tariq et al., 2025). The soil is a sandy loam of 33.4% coarse sand, 31.9% fine sand, 16.8% silt, 13.3% clay, and 4.6% humus. Chemical fertilizer and pig slurry, with and without NI were added to field plots on April 20, 2023, and same treatments were applied in 2022 (Supplementary Table S1). Soil sampling was conducted after eight days in spring and again after five months in fall, specifically on April 28th and August 18th (Supplementary Table S1). We sampled a total of 30 experimental plots with the following 10 treatments: (1) negative control with no fertilizer application, (2) pig slurry at 153 kg N ha^{-1}, (3) pig slurry at the recommended dosage of the nitrification inhibitor Vizura® (EuroChem), (4) pig slurry with 3× the recommended dosage of Vizura, (5) pig slurry with 10× the recommended dosage of Vizura, (6) pig slurry at recommended dosage of the nitrification inhibitor Instinct® (Corteva Agriscience, Copenhagen, Denmark), (7) pig slurry with 3× the recommended dosage of Instinct, (8) pig slurry with 10× the recommended dosage of Instinct, (9) chemical fertilizer of ammonium sulphate nitrate (total N 26.0% (NO_3^-—N 7.5%: NH_4^+—N 18.5% and S 13.0%), and (10) Same fertilizer as (9) but with 3,4-dimethylpyrazole phosphate (DMPP) coated granulates (ENTEC®, BASF, Ludwigshafen am Rhein, Germany) (Table 1). Vizura consists of DMPP and Instinct consists of nitrapyrin, and the recommended dose for DMPP is applied at 2 kg ha^{-1} and NP at 2.5 kg ha^{-1}. Each plot consisted of a 3 m × 3 m area, and for the test of NI concentrations, each plot was divided into four subplots, where the first subplot was undisturbed, and the remaining three (1×, 3×, and 10 x recommended dose of NI) were sampled in this study.

Soil samples were collected from each plot using 20 cm soil cores with a diameter of 2.5 cm. In each plot, 5 soil cores were collected following a *Z*-shaped pattern. For the subplots, cores were taken from three subplots out of the four 1.5 × 1.5 m^2 subplots, whereas the fourth subplot was undisturbed. Soil cores collected were then thoroughly mixed in a plastic bag, from which approximately 40 g of soil was

Table 1
Field experimental setup using RCBD design using two fertilizers and two nitrification inbibitors. Abbreviations: C (control), NS (chemical fertilizer NS), NE (chemical fertilizer NS + ENTEC), PS (pig slurry), PV (pig slurry + Vizura), PI (pig slurry+Instinct),. [1]: 26–13: 26%N and 13%S; [2]: 1×, 3×, and 10×: 1-, 3-, and 10-times recommended concentration of NI.

Fertilizer Type	NI Used	NI Product	Manufacturer / Details	Abbreviation
None	–	–	Negative control with no fertilizer application	C
Chemical fertilizer	–	NS (26–13)[1]	26.0% N (7.5% (NO_3^--N:18.5% NH_4^+-N, 13.0% S	NS
	DMPP	ENTEC®	Same as NS (26–13), granulate coated with DMPP (BASF)	NE
Pig slurry	–	–	153 kg N ha^{-1}	PS
	DMPP	Vizura® (1×)[2]	EuroChem; DMPP = [X] g/L	PV 1×
		Vizura® (3×)[2]	EuroChem; DMPP = [3 × X] g/L	PV 3×
		Vizura® (10×)[2]	EuroChem; DMPP = [10 × X] g/L	PV 10×
	Nitrapyrin	Instinct® (1×)[2]	Corteva Agriscience; Nitrapyrin = [Y] g/L	PI 1×
		Instinct® (3×)[2]	Corteva Agriscience; Nitrapyrin = [3 × Y] g/L	PI 3×
		Instinct® (10×)[2]	Corteva Agriscience; Nitrapyrin = [10 × Y] g/L	PI 10×

sampled into 50 mL Falcon tubes. The Falcon tubes were immediately frozen in liquid nitrogen in the field and subsequently stored at −80 °C in the lab before undergoing DNA and RNA extraction. Soil cores were mixed in plastic bag, moved to falcon tubes and frozen in liquid nitrogen within 5 min of sampling.

2.2. Co-extraction of eDNA and eRNA from soil

Before nucleic acid extraction, soil samples were initially lyophilized using a freeze dryer (Scanlaf Model Coolsafe 55 Lynge, Denmark) for at least 72 h to preserve nucleic acid as recommended earlier (Weißbecker et al., 2017). The samples were then subjected to homogenization in a bead mill homogenizer (Bead Ruptor Elite, Omni International) using 2.4 mm metal beads (Metal Bead Media, Omni International, USA) at 4 m s^{-1} speed for 30 s, repeated three times. This homogenization step allowed mixing and grinding of approx. 40 g of soil in Falcon tubes. Soil DNA and RNA co-extractions of a subsample of 0.25 g of the ground soil were carried out using the Nucleobond RNA soil Mini Kit (Macherey-Nagel; Germany) according to the manufacturer's instructions. Following RNA extraction, DNA was subsequently extracted from the same sample using the DNA set for Nucleobond RNA soil Mini Kit (Macherey-Nagel; Germany). This kit has been successfully shown to extract high-quality RNA, including mRNA, from soil samples from different environments (Hurt et al., 2001; Scheel et al., 2023). All the DNA/RNA extractions were quantified with the Qubit kit (1× dsDNA HS assay and Qubit RNA HS Assay) using Qubit 4.0 Fluorometer (Invitrogen, Oregon, USA). The extracted RNA was treated with DNase using the RapidOut DNA Removal Kit (Thermo Fisher Scientific). Following DNase treatment, both RNA and DNA concentrations were quantified using a Qubit 4.0 Fluorometer to verify the absence of residual DNA. The mean concentration of DNA in DNase treated RNA was mean 0.35 ng/ul, likely to represent the degraded DNA fragments. To further confirm the absence of amplifiable residual DNA, 18 randomly selected DNase treated RNA samples were used for PCR as no-RT PCR control. We found no amplification in the no-RT controls. Subsequently, the extracted DNase treated RNA was transcribed into complementary DNA using the First Strand cDNA Synthesis Kit (Roche) before being used for amplicon PCR.

2.3. Amplicon library preparation

We used universal primers mlCOlintF (GGWACWGGWTGAACWGTWTAYCCYCC) and jgHCO2198 primers (TANACYTCNGGRTGNCCRAARAAYCA) targeting the COI region of the metazoan mitochondrial genome (Leray et al., 2013), similar for both DNA and RNA amplification. A two-step PCR dual-indexing strategy for Illumina MiSeq sequencing was used. Each primer had Illumina Nextera overhang adapters attached. During the first PCR (PCR#1), amplicons were generated using 0.25 µl HiFi polymerase (PCRBiosystems), 5 µl HiFi buffer, 0.5 µl 10 µM of each forward and reverse primer, 0.5 µl BSA, 2 µl template and nuclease-free water to a total volume of 25 µl. The PCR#1 thermocycler program included an initial denaturation temperature of 95 °C for 5 min, then ten cycles of 94 °C for 30s, 50 °C for 30 s, 72 °C for 1 min, another 10 cycles of 94 °C for 30s, 52 °C for 30 s, 72 °C for 1 min, followed by 15 cycles of 94 °C for 30s, 54 °C for 30 s, with the final elongation at 72 °C for 2 min. For dual indexing, the second PCR (PCR#2) included 5 µl of PCR#1 product and 2 µl of primers with Illumina adaptors and indexes with the PCR reaction described above. The PCR#2 program included 98 °C for 1 min, then 13 cycles of 98 °C for 10 s, 55 °C for 20 s, and 68 °C for 40 s, and a final elongation of 68 °C for 5 min. Negative controls (no-template controls, NTCs) were included during library preparation to monitor potential contamination. The PCR amplification was confirmed by running 1.5% agarose gels using SYBR green staining. The PCR#2 products were cleaned for primer dimers using 20 µl HighPrep™ magnetic beads (MagBio Genomics Inc. Gaithersburg, Maryland, USA) according to the manufacturer's instructions and eluted in 25 µl TE buffer. Finally, DNA concentrations were measured using Qubit 4.0 (Thermo-Fischer Scientific) and the High-Sensitivity DNA assay kit. Samples were then equimolarly pooled and sequenced on an Illumina MiSeq using the 500 cycles V2 reagent kit at the Department of Environmental Science, Aarhus University.

2.4. Bioinformatics

Sequencing data from Illumina MiSeq reads were analyzed using vsearch (Rognes et al., 2016). Before downstream analysis, reads were truncated for primer sequences for both forward and reverse reads, along with removing low-quality reads (<q20) and reads less than 100 bp using cutadapt (Martin, 2011). Then, paired-end reads were joined with an overlapping minimum read length of 30 base pairs, followed by quality control, chimera detection, dereplication, and clustering using vsearch. Clustering was done at 99% similarity level, and singletons were discarded. OTUs were blasted manually using the sequence-id tool available (www.gbif.org/tools/sequence-id) against the BOLD database (v.2024-07-19) (Ratnasingham and Hebert, 2007). All the OTUs assigned to Animalia within the phyla Annelida, Arthropoda, Nematoda, Tardigrada, Mollusca, and Rotifera were retained, and the rest were discarded. In addition, taxonomic classification of these OTUs was performed against the MIDORI database for COI using RDP classifier (Machida et al., 2017), and only those OTUs assigned with a confidence level of more than 50% at the phyla level were retained, and the rest were discarded. For the GBIF BLAST output, taxonomy assignments were further curated based on a minimum coverage and identity threshold of >97% for species, >95% for genus, >90% for family, >85% for order, >80% for class, and > 70% for phylum level.

2.5. Data and statistical analysis

The resulting taxonomy and OTU tables were exported into R ver. 4.3.1 (R Core Team, 2023) for data analysis and visualization. Data wrangling and diversity-based analyses were done using the '*vegan*' package ver. 2.5–7 (Oksanen et al., 2020) and the '*phyloseq*' package ver.

1.34 (McMurdie and Holmes, 2013). Samples less than 100 reads were removed which led to removal of eight eRNA spring samples comprising four chemical fertilizers, two pig slurry and two control samples. Alpha diversity was estimated using richness, while beta diversity was calculated using Sørensen dissimilarity matrices. To ensure robust estimates, OTU table was rarefied 1000 times, and the mean of the resulting diversity estimates was used. Pairwise difference was assessed using the Wilcoxon rank-sum test, with *p*-values adjusted using the Benjamini–Hochberg method. Distance matrices were visualized using principal coordinate analysis (PCoA). PERMANOVA was conducted using the 'adonis' test from the 'vegan' package to test the effect of fertilizer, eDNA vs eRNA, NI and NI concentrations on the community structure. Since the experiment was a randomized complete blocks design (RCBD) and its split-plot structure for the within-plot four levels of NI applications, we used blocks as a random effect, whereas fertilizer was a whole plot factor and NI applications levels as sub-plot factor in the 'adonis' test. To identify the OTUs associated with NI concentrations, we used NMDS ordination on presence-absence data using Sørensen dissimilarity. We applied the envfit() function, fitting OTU vectors and environmental variables onto the NMDS ordination with 999 permutations. For OTUs showing significant association, prevalence was calculated as the proportion of samples in which the OTU was present. The number of shared vs unique OTUs were visualized using UpSet plot with the *UpSetR* package v1.4 (Conway et al., 2017).

3. Results

3.1. Data characteristics

In this study, we present eDNA- and eRNA-based metabarcoding data derived from soil. Environmental DNA (eDNA) was directly extracted from soil, and a total of 60 soil samples were targeted using both eDNA and eRNA approaches. Amplicon sequencing 120 (60 + 60) samples after quality filtering, subsetting to the phyla Annelida, Arthropoda, Nematoda, Tardigrada, Mollusca, and Rotifera and OTUs assigned with a confidence level > 50% at the phyla level yielded 750,799 reads, which were assigned to 1386 OTUs. Overall, we obtained 6256 ± 7326 (mean ± standard deviation) reads per sample. In general, higher number of reads resulted in eDNA samples from both spring (11,666 ± 5787) and fall (18,074 ± 10,166) compared to eRNA samples from spring (1309 ± 1455) and fall (11,053 ± 8913).

3.2. Comparison of season shifts in community structure

Soil fauna communities were clearly separated based on sampling season (spring vs fall) and based on nucleic acid extraction (RNA vs DNA) (Fig. 1). Species richness was compared on the overall dataset across the two nucleic acids, RNA and DNA, using observed (richness) and Shannon diversity indices. eDNA metabarcoding captured higher alpha diversity measured by both observed richness. eDNA showed higher diversity in the spring, while eRNA showed higher diversity in the fall (Fig. 1A). This indicates the growth of a fraction of the population and a higher diversity of active soil fauna in the fall. Principal coordinate analysis (PCoA) plot using Sørensen dissimilarity distances showed clear clustering based on the two seasons and nucleic acid type (eRNA and eDNA) (Fig. 1B). A permutation multivariate analysis of variance (PERMANOVA) showed that the nucleic acid (eRNA vs eDNA) explained a large part of the variation (25%) followed by the season (4%). By sub-setting data sets based on eRNA or eDNA, sampling season was the dominating factor driving the community structure. Interestingly, the season explained a larger part of the variation (12%) in eRNA metabarcoding compared to eDNA metabarcoding (7%). In addition, the effect of fertilizer was consistent across the two methods (Table 2).

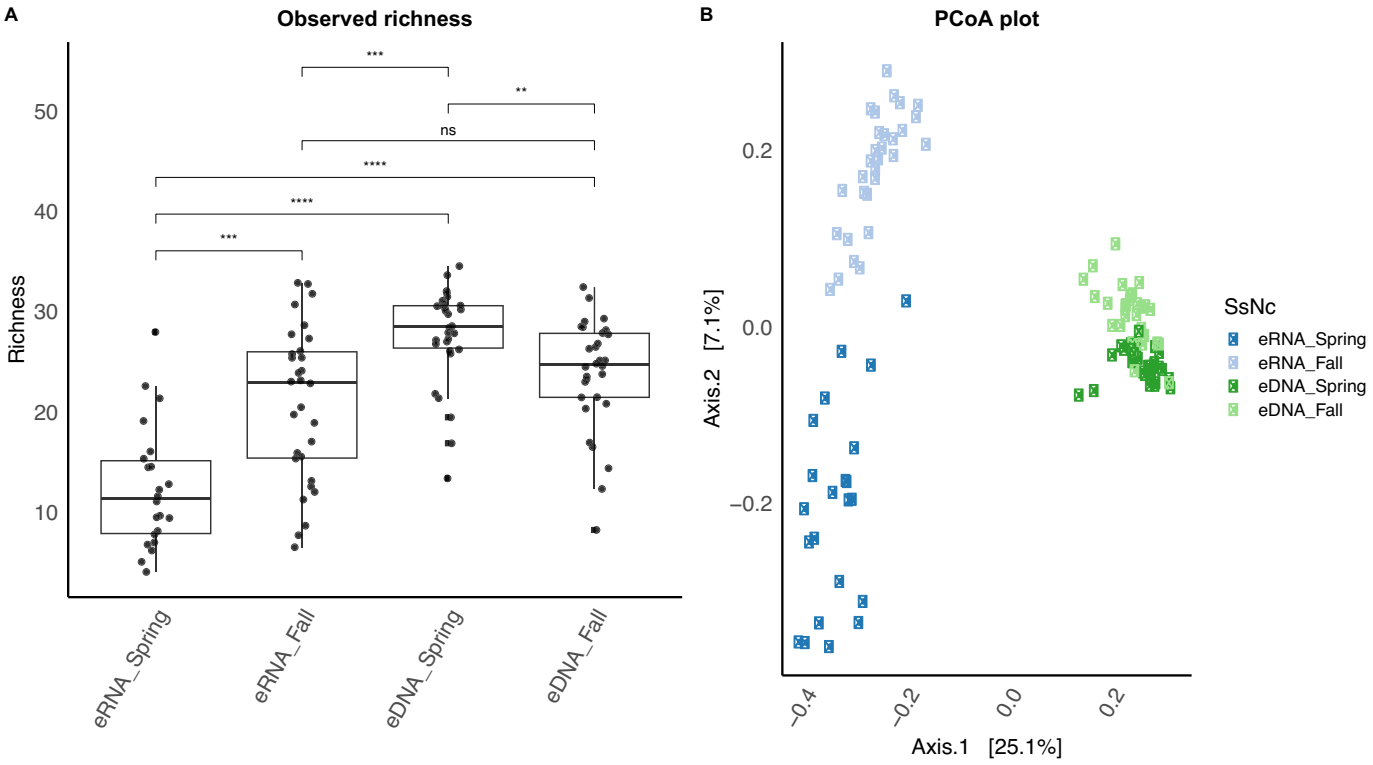

Fig. 1. Comparison of soil fauna alpha and beta diversity between DNA and RNA-based metabarcoding of soil samples. A) Boxplot showing the invertebrate OTU richness (observed) obtained by metabarcoding of eRNA and eDNA. Pairwise comparisons were assessed using Wilcoxon rank-sum test; adjusted *p*-values are indicated as follows: * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$. B) Principal coordinate analysis (PCoA) of the soil fauna Sørensen's dissimilarity matrix across two seasons using eRNA and eDNA metabarcoding.

Table 2
Results of PERMANOVA using adonis test based on Sørensen distance matrices for community dissimilarity assessment using 1000 permutations. The effect of block was incorporated in the adonis test to correct the partitioning of variance. *: $p > 0.05$; ***: $p < 0.001$.

Dataset	Factor	PERMANOVA R2
Whole	Season	0.04***
	Method (eRNA vs eDNA)	0.25***
eRNA	Season	0.12***
	Fertilizer	0.05*
eDNA	Season	0.07 ***
	Fertilizer	0.04 ***

3.3. Comparing OTUs taxonomy via eRNA and eDNA

We found a large overlap of taxa captured by both eRNA and eDNA across two seasons (Fig. 2). Interestingly, insects dominated the number of OTUs shown by the size of the bubble. eRNA spring data showed the lowest number of insects OTUs visualized by smallest size of bubble, and a similar trend was seen in Collembola and Arachnida. Except Symphyla within Arthropoda, all other taxa were shared across two seasons by eRNA and eDNA methods. Interestingly, a large overlap of OTUs was observed for Clitellata (Annelida) which consists of annelid worms such as earthworms.

For an overview of shared vs unique OTUs, we used an upset plot across season and nucleic acid types. When evaluated across seasons, it showed seasonal differences between eDNA and eRNA, with eDNA having the highest number of OTUs in spring, while in the fall the shared OTUs between eDNA and eRNA was the larger fraction of OTUs (Fig. 3A and B). Similarly, Upset plots based on nucleic acids showed that shared OTUs across seasons dominated the eDNA dataset, whereas the eRNA was dominated by unique OTUs in fall, followed by shared OTUs as the second most abundant (Fig. 3 C and D). Further, comparing shared OTUs in all four datasets (across season and nucleic acid), we found that eDNA both fall and spring shared the highest number of OTUs, followed by eRNA fall (Supplementary Fig. S1). eDNA fall showed a higher number of unique OTUs, followed by eDNA spring, eRNA fall, and with no shared OTUs in eRNA spring.

The relative read abundances of soil fauna showed differences in eRNA and eDNA. Both datasets revealed clear dominance of Insecta, Collembola, and Clitellata. While the dominance of Insecta was clearly seen in the eDNA dataset, Clitellata dominated the eRNA dataset

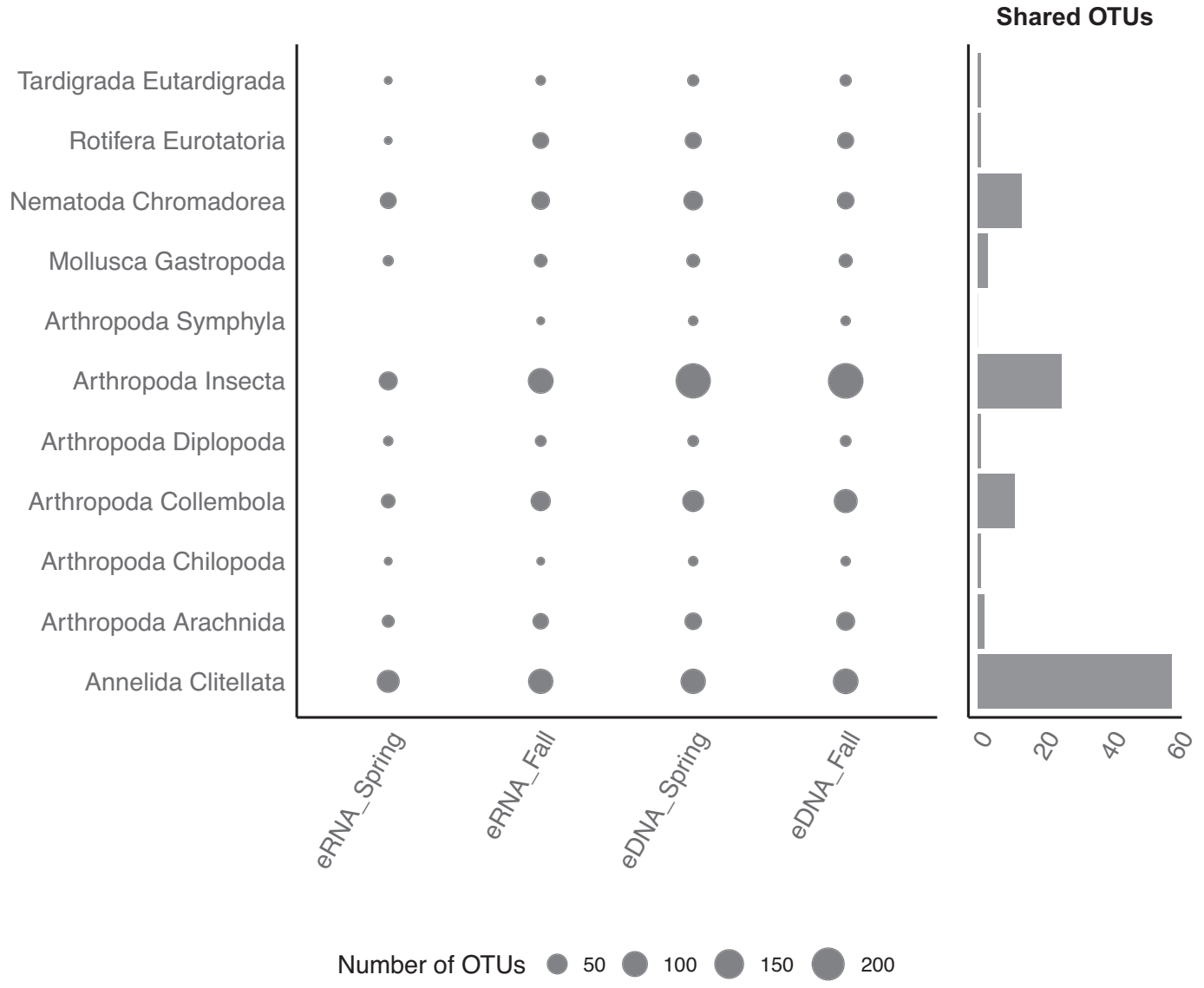

Fig. 2. Bubble plot showing number of invertebrate OTUs detected over the invertebrates' order using eRNA and eDNA methods. The size of the bubble represents the number of OTUs.

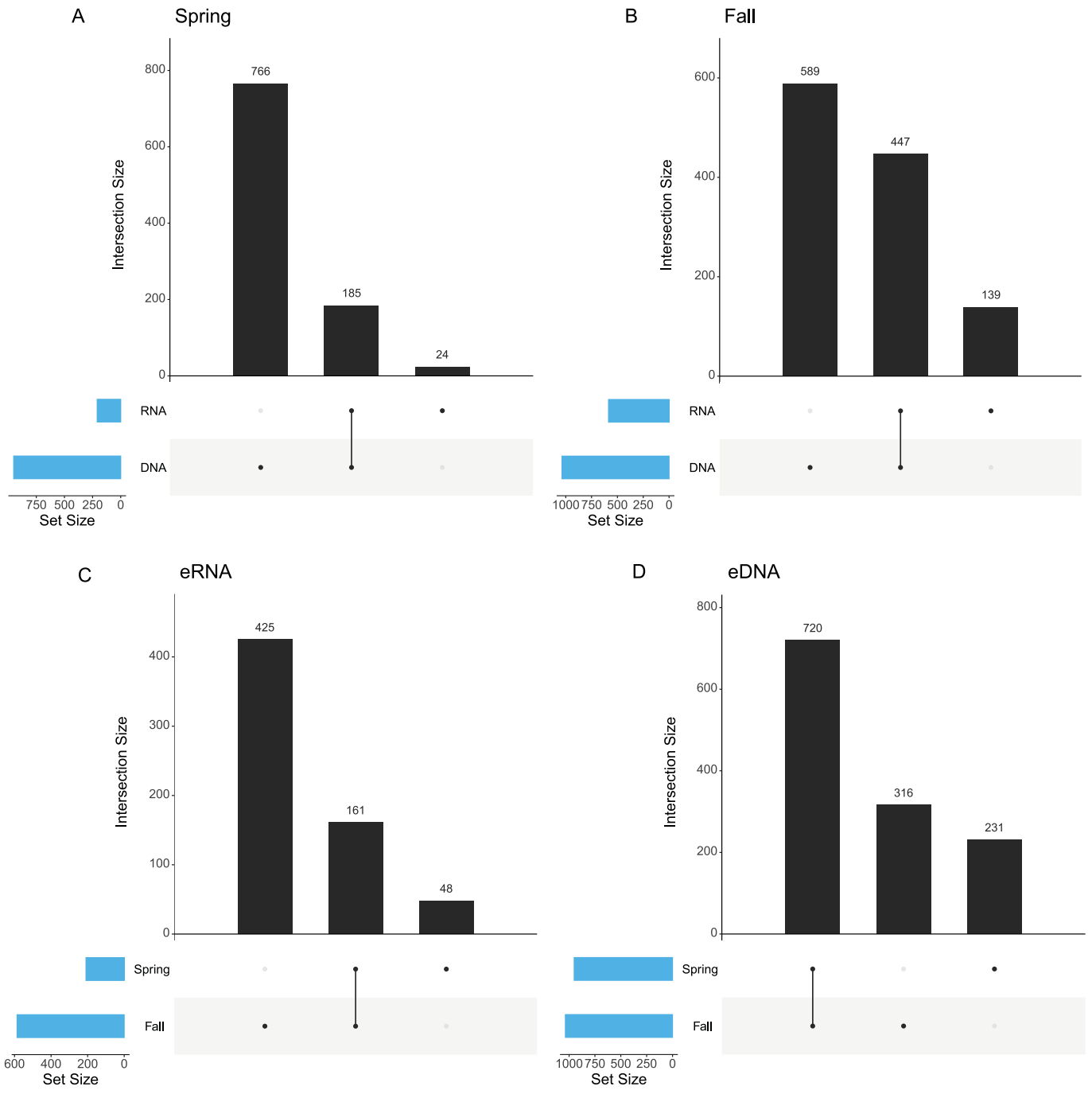

Fig. 3. UpSet plot showing shared vs unique OTUs among the two nucleic acid extraction methods (eDNA and eRNA) from the two seasons: Spring (A), Fall (B), eRNA (C), and eDNA(D).

(Fig. 4).

3.4. Effects of fertilizer and NI

Data was split based on nucleic acid and season to test for the effect of fertilizer. We found that the fertilizer effect was significant in both eRNA- ($p < 0.05$) and eDNA-based ($p < 0.001$) metabarcoding data (Table 2). Further, we subsetted the data for pig slurry (PS) treatment to test the effects of NI concentration, as multiple NI concentrations (including the control) were included for PS, whereas such NI concentration gradients were not part of the chemical fertilizer treatment. The effect of NI concentration was exclusively detected in the spring eRNA dataset using adonis test ($R2 = 0.12$, $p < 0.05$) and manyglm test ($p < 0.05$). Given the significant effect observed in the spring PS eRNA dataset, it was used to identify the most NI-affected OTUs. Among the eight OTUs identified by the envfit() function, we found six being Arthropoda (OTU_47, 193, 335, 885, 658, 856), followed by three OTUs (OTU_8, 44, 12519) from Annelida and one OTU assigned to Nematoda (Fig. 5, Table 3).

Using split data, we investigated the effect of the two different NIs on soil fauna richness. We only found significant effect observed in the fall eRNA subset data, where nitrapyrin showed significantly lower diversity compared to DMPP. We found a similar trend in eDNA dataset, however, it was not significant (Fig. 6).

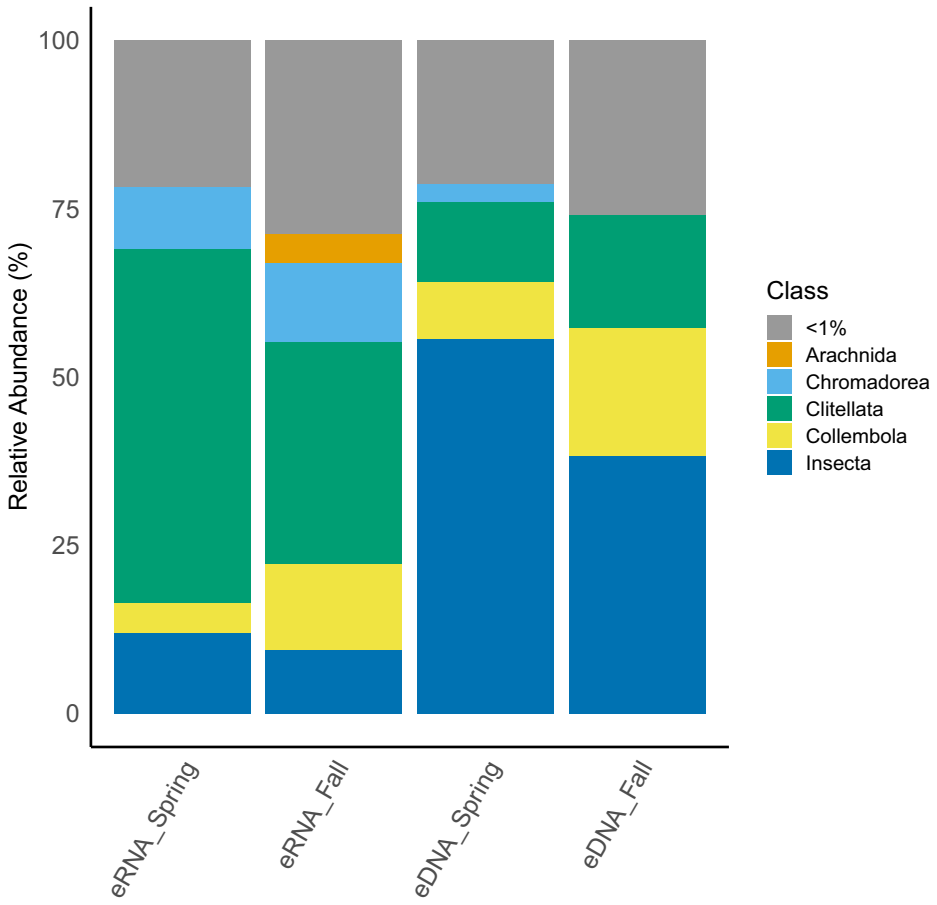

Fig. 4. Composition of soil fauna communities at class level in soils from two seasons and two nucleic acid extraction metabarcoding methods. Class with relative abundance of <2% in each subplot were assigned to "<2%".

4. Discussion

In this study, we present the first application of eRNA COI metabarcoding in soil ecotoxicology, exemplified through the assessment of effects of fertilizer and NIs on soil fauna, along with eDNA COI metabarcoding. eDNA has been widely applied in monitoring soil organisms, including invertebrates, primarily due to its stability and persistence in environmental samples such as soil (Kirse et al., 2021a; Winding et al., 2019). However, this stability becomes a limitation when the goal is to monitor the active communities in the soil. Our results show that the two molecular approaches (based on eRNA and eDNA COI metabarcoding) provide a complementary picture of soil biodiversity. Both methods captured diverse soil fauna and showed distinct community patterns. However, each method differed in sensitivity and resolution. We found eRNA metabarcoding was more effective in tracking non-target impacts of NIs on soil fauna communities in arable soils cultivated with spring barley across seasons compared to eDNA metabarcoding. Such observations indicate that eRNA, due to its rapid degradation (Kagzi et al., 2022), provides a snapshot of biological activity, whereas eDNA may represent both relic and living organisms due to its slower degradation in soil (Kirse et al., 2021b). Our findings highlight the benefits of using eRNA as a tool for activity-based biomonitoring and its potential for biomonitoring of soil fauna in the cultivated field, especially when evaluated alongside eDNA.

eRNA instability contributes to its wider use in microbial ecology through approaches such as total RNA sequencing, which includes rRNA and mRNA (Bang-Andreasen et al., 2020) or quantitative PCR targeting mRNA for specific gene expressions. Besides the concern on whether the degradation rate of eRNA could impact the detection of arthropods in freshwater systems (Kagzi et al., 2023), eRNA is consistent with earlier results in the microbial systems and strengthens the argument for eRNA as a powerful proxy for living communities (Yates et al., 2021). While the concept of eRNA for arthropods has been shown in an aquatic environment, where eRNA showed temporal dynamics of zooplankton (Ankley et al., 2022), and eRNA for microbes works in soil (Adamo et al., 2020; Bang-Andreasen et al., 2020), our results show that eRNA sequencing analyses works for higher multicellular organisms like arthropods in the soil environment.

As previous studies of OTU vs amplicon sequence variants (ASVs) based workflows, including studies using protein-coding genes such as COI, indicated that OTU clustering is still valid, and can mitigate artefactual sequence inflation (Antich et al., 2021; Glassman and Martiny, 2018), we used OTU-based clustering which accounts for technical variability associated with additional reverse transcriptase steps during library preparation.

OTUs were clustered at 99% sequence similarity instead of 97% threshold commonly used in microbiome studies. Given that the COI region amplified using Leray primers is approximately 313 bp long, the 99% threshold allows up to three nucleotide differences within an OTU, which likely accommodates low-frequency, stochastic errors if introduced during reverse transcription (Verwilt et al., 2023). Based on these OTUs, the overlap of OTUs between eRNA and eDNA datasets was calculated.

The temporal dynamics offered by eRNA were evident in our seasonal comparisons. Both eDNA and eRNA methods revealed distinct clustering of community structure, as shown by PCoA plots and supported by PERMANOVA analyses. However, eRNA showed a more dispersed distribution, suggesting higher sensitivity to seasonal variation. Our results show that both eDNA and eRNA can resolve these temporal dynamics and provide complementary understanding of the

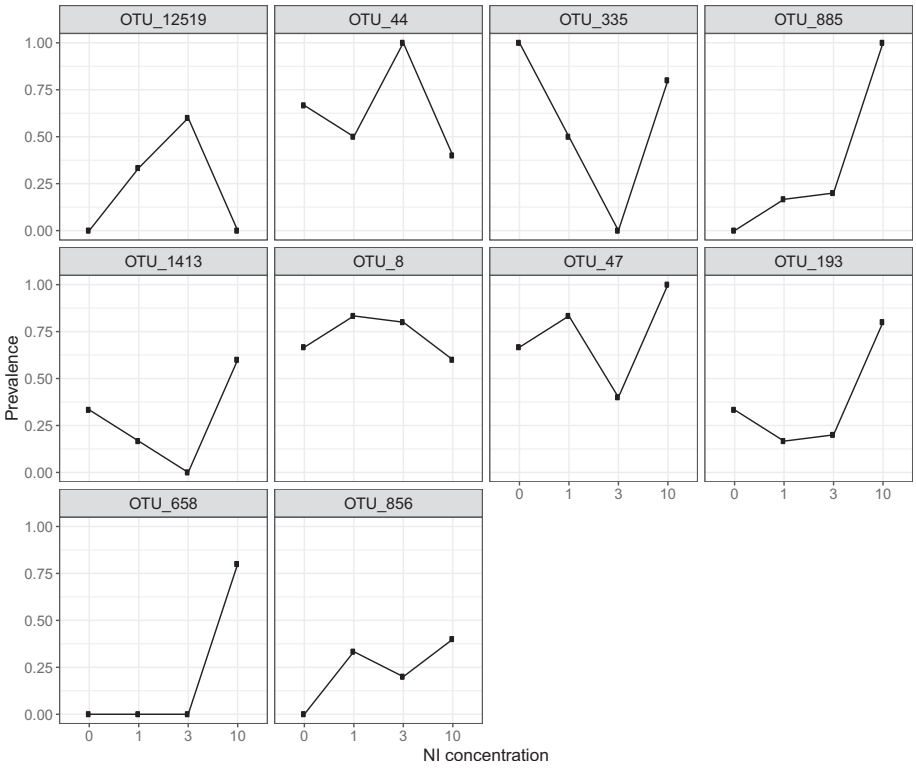

Fig. 5. Prevalence of significant OTUs in spring eRNA 2023 in response to NI concentrations. Significant OTUs identified by Sørensen-based NMDS and envfit() function, and prevalence was calculated as the proportion of samples in which the OTU was present using present-absent data. Lines connect the prevalence values across concentration to show OTU occurrence pattern. The taxonomy of each OTU is presented in Table 3.

Table 3
Taxonomy of top 10 important features (OTUs) associated with the NI concentration.

OTU	NMDS1	NMDS2	pval	r2	Taxa
OTU_44	−0.843	−0.538	0.001	0.662	Annelida;Clitellata; Clitellata;Crassiclitellata; Lumbricidae; Aporrectodea
OTU_12,519	−0.773	−0.635	0.002	0.587	Annelida;Clitellata; Clitellata;Crassiclitellata; Lumbricidae; Aporrectodea
OTU_335	0.885	0.466	0.004	0.508	Arthropoda
OTU_1413	0.496	−0.868	0.01	0.466	Nematoda;Chromadorea; Chromadorea;
OTU_47	0.974	−0.224	0.011	0.438	Arthropoda
OTU_193	0.755	−0.656	0.012	0.453	Arthropoda
OTU_8	−0.500	0.866	0.013	0.412	Annelida;Clitellata; Clitellata;Crassiclitellata; Lumbricidae; Aporrectodea
OTU_885	0.646	−0.763	0.013	0.439	Arthropoda
OTU_658	0.866	−0.500	0.018	0.389	Arthropoda;Collembola; Collembola; Entomobryomorpha
OTU_856	0.634	0.773	0.026	0.375	Arthropoda

variations.

Monitoring of soil fauna diversity is mainly carried out during spring and fall, as season shapes the activity and abundance of soil organisms in temperate agricultural systems (Ball et al., 2026; Petersen, 2000; Rutgers et al., 2016; Santorufo et al., 2014). Our results on eRNA-based metabarcoding also follow this trend, where diversity is higher in the fall compared to spring. However, it is vital to interpret our findings within the methodological limitations of eRNA metabarcoding. While eRNA supports the fact that fall has more active fauna, the approach primarily captures the presence of genetic signals rather than a direct measure of metabolic activity. Therefore, the observed seasonal differences in eRNA diversity are more likely driven by increased population sizes and higher detectability of taxa in autumn rather than higher physiological activity per se.

In contrast, eDNA-based metabarcoding showed less seasonal variation, highlighting the stability of eDNA-based community structure. Our findings on seasonal relationships captured via eRNA and eDNA indicate the benefit of eRNA for ecological monitoring, particularly for the temporal shifts in soil fauna and functional aspects of soil fauna in response to environmental changes.

Notably, eDNA metabarcoding recovered significantly higher taxonomic richness of soil fauna compared to eRNA, likely due to its ability to capture a combination of living and dead organisms, including the dormant life forms (Pochon et al., 2017). Higher species richness of the eDNA record can be beneficial when the study aims at a comprehensive inventory of biodiversity, particularly when rare or dormant taxa need to be included. However, this inclusivity also raises concerns of overestimation of active members of the community. In contrast, the activity-linked profile offered by eRNA increases the utility for identifying the immediate biological response. Together, our findings have important implications for soil biodiversity studies, illustrating how DNA and RNA metabarcoding are complementary by distinguishing active from legacy communities, which is essential for understanding ecological processes.

Interestingly, our results showed the exclusive detection of certain OTUs in either eRNA or eDNA datasets in addition to a large overlap. OTUs only found in the eRNA dataset might thus represent metabolically active, short-lived, or transient members of the soil community, performing functionally important contributions but often overlooked by the eDNA-based approach (Yates et al., 2021). On the other hand, eDNA-only taxa are likely to cover legacy or dormant organisms, contributing to a broader but temporally less resolved picture of biodiversity. Further, the persistence of OTUs across seasons in eDNA, and dominance of

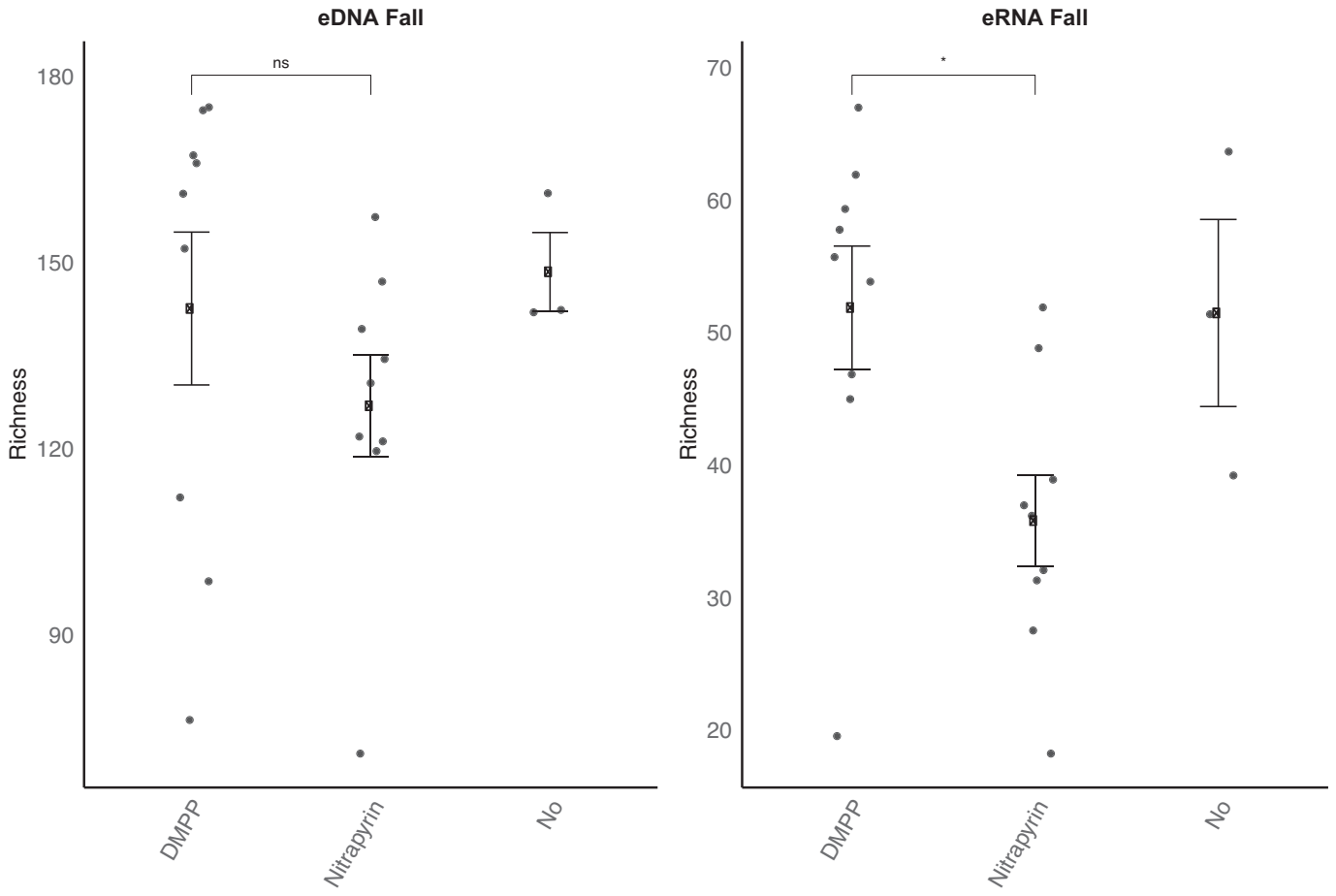

Fig. 6. Comparison of soil fauna richness between different nitrification inhibitors (NIs). Plots showing the invertebrate OTU richness (observed) obtained by metabarcoding of eRNA and eDNA. Pairwise comparisons were assessed using Wilcoxon rank-sum test; adjusted p-values are indicated as follows: * = $p < 0.05$, and ns for non-significant.

unique OTUs in the fall eRNA data, supported by our upset plots, indicate that DNA-based profiles predominantly capture a stable core community shared across seasons, whereas eRNA more strongly reflects season-specific and potentially active taxa. A similar comparative study conducted in aquatic environments showed that eRNA enabled more accurate detection of living species in lakes compared to eDNA (Littlefair et al., 2022).These differentiations between active and legacy members are useful for gaining insights into the functional and structural dimensions of soil fauna communities. However, dominance of unique OTUs in the fall eRNA pattern should be interpreted with caution, as, although samples were equimolarly pooled, the spring eRNA samples had a relatively low number of reads per sample after quality control and selection for the target soil fauna taxa, which may have contributed to the apparent enrichment of unique OTUs in the fall eRNA dataset. Furthermore, the integration of eRNA and eDNA metabarcoding with direct observations or experimental manipulations could help us to confirm the roles of specific taxa and further validate their activity status.

Importantly, the availability of environmental variables in our experiment allowed us to test how the community responds under different treatments. After splitting our data based on nucleic acid type and season, we assessed the effects of fertilizer and NI application on soil fauna communities. Overall, the effect of fertilizer was significant on the eRNA and eDNA datasets. When we focused on the effects of the NI concentration in the pig slurry treatment, NI concentrations had no detectable effect on the spring or fall eDNA datasets, but eRNA data from the spring showed a significant effect of NI concentration. The detection of NI effect in spring eRNA suggests a season signal; however, direct causal effect should be made cautiously. It could be linked to timing of NI application relative to sampling, and with the degradation or leaching of NIs in soil with the effectiveness diminishing later in the season, as reflected in temporal patterns of N_2O emission rates (Peixoto and Petersen, 2023). We observed the NI effect eight days after NI application, which may be linked to the period of NI being biologically active and affecting the soil organisms. Further, such signal also suggests that eRNA is more sensitive to detect subtle short-term effects of biological responses to environmental change (Greco et al., 2022). NIs alter the microbial activity of ammonium-oxidizing bacteria (AOB) and archaea (AOA) (Liu et al., 2015; Subbarao et al., 2006), which could lead to downstream changes in the soil fauna that feed or rely on some of the microbial processes.

Interestingly, the effect of two different NIs (Nitrapyrin and DMPP) were only observed in the fall eRNA without any effect of the NI concentration. This likely reflects the degradation or leaching of NI over time, thus removing it from the soil. Similarly, though showing a similar trend, the absence of significant effects of two NIs for the eDNA may be due to legacy eDNA in spring that might capture DNA from the previous year and overshadow the recent effect of NI application.

5. Conclusion

In this study, we highlight the complementary roles of eRNA- and eDNA-based metabarcoding for soil fauna monitoring. Our results demonstrate that eRNA-based community profiles reflect metabolically active organisms, offering a temporally resolved snapshot of biodiversity that is not confounded by relic or extracellular DNA. While this is

reported in aquatic systems, this is the first time reported in the soil environment. The seasonal changes observed in RNA-based communities show their strength and potential not only for ecological monitoring but also for detecting the functional response of soil fauna to anthropogenic and environmental changes. The fertilizer effect detected both in the eRNA and eDNA data suggests a consistent impact on soil fauna communities, whereas the limited NI concentration effect, observed only in the spring dataset, may reflect seasonal interaction among NI concentration, microbial activity, and soil fauna dynamics. Further, our findings support the use of eRNA-based metabarcoding for soil fauna communities and emphasize the importance of differentiating between active vs total species signal in biodiversity assessments. Overall, our findings position eRNA-based metabarcoding as a vital addition to soil biodiversity assessment of soil fauna, highlighting its potential to link community composition to ecosystem processes. Future studies combining eRNA from soil with environmental and experimental data will further improve our ability to predict soil fauna response to anthropological effect in soil ecosystem monitoring.

CRediT authorship contribution statement

Rumakanta Sapkota: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yuan Pan:** Writing – review & editing, Methodology, Data curation. **Vid Naglič:** Writing – review & editing, Methodology, Data curation. **Lea Ellegaard-Jensen:** Writing – review & editing, Methodology, Investigation. **Paul Henning Krogh:** Writing – review & editing, Supervision, Investigation, Data curation, Conceptualization. **Anne Winding:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

Acknowledgments

The study was supported by the Danish Climate Research Program, project KLIMINI grant no. 33010-NIFA-19-726 and the eDNA Center at Technical Sciences, Aarhus University. We would like to express appreciation to the laboratory technicians Tanja Begovic and Tina Thane, and PhD students Julie Egelund Andersen and Zivile Buivydaite for their assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2026.106996.

Data availability

The sequence data has been deposited in the NCBI Sequence Read Archive (SRA) database and can be accessed under the BioProject ID PRJNA1284630.

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