European Journal of Soil Biology 115 (2023) 103474

Contents lists available at ScienceDirect

European Journal of Soil Biology

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

Original article

Earthworms shape prokaryotic communities and affect extracellular enzyme activities in agricultural soil

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ARTICLE INFO

Handling Editor: Anton Potapov

Keywords:
Bacteria
Archaea
Glucosidases
16S rRNA genes
Metabarcoding

ABSTRACT

Earthworms are known for their important role in soil ecosystem functioning and are used as indicators of ecosystem health. Earthworms and soil microorganisms are major players in soil ecosystem processes. However, understanding of their interactions is limited. Using microcosms, we studied the effect of earthworms on soil microorganisms in entire soil mesocosms by comparing soil with and without earthworms. Soil microbial activity was determined by an extracellular enzyme activity assay, while soil DNA was used to determine prokaryote abundance by quantitative PCR targeting 16S rRNA genes and community composition and diversity by amplicon sequencing of 16S rRNA genes. The microbial activity showed an indication of increase of chitinase, α-glucosidase, β-glucosidase and endo-β-glucanase during incubation with a specific increase in endo-β-glucanase activity in the presence of earthworms. Importantly earthworms decreased species richness ($p = 0.002$) and were a significant factor ($p = 0.008$) in shaping soil prokaryotes community structure. Moreover, our results revealed enrichment of bacterial phyla of *Bacteroidetes* and *Proteobacteria*, as well as reduction in relative abundance of the archaeal phylum *Thaumarchaeota,* suggesting that the presence of earthworms favors specific microbes in soil. Further, differential abundance analysis showed strong correlations between enzymatic activities (all tested except phosphomonoesterase) and relative abundances of specific bacterial OTUs. Our findings suggest that earthworms influence the soil microbial communities and their activity in soil, and hence earthworm-prokaryote interactions should be incorporated in future soil microbiome studies.

1. Introduction

Earthworms are well known for their importance to soil health due to their effects on soil structure and nutrient cycling (reviewed in [55]). Earthworm induced effects on soil microorganisms are mediated by their feeding and burrowing activities which alter soil properties and structures. Consequently, earthworms affect chemical, physical and biological processes in the soil [55,56].

In addition, earthworms indirectly impact soil properties as excreted intestinal mucus and the casts serve as an energy source for soil microorganisms [1] and thus modifies microbial community composition in the surrounding soil [57]. The earthworm gut itself creates a special habitat for microorganisms by creating constant source of moisture, readily available nutrients and limited oxygen, and the earthworm gut has been shown to harbor distinct bacterial communities compared to bulk soil [2]. The part of the soil influenced by earthworms, the drilosphere, has also been identified as a hotspot of microbial activity [3–5]. Both prokaryotes, fungi, protists and nematodes were found to be affected by earthworm activities, in particular in the burrow walls which hosted a higher microbial biomass, activity and diversity than bulk soil [6–8].

Furthermore, microorganisms affect soil biochemical cycles, partly through extracellular enzyme activities. These enzymes are essential in global nutrient cycling and litter decomposition and are useful indicators of soil functioning and fertility as their measurements incorporate information on both the microbial status and the biochemical soil potential [9,10]. Earthworms have been found to modify the microbial enzyme activities in earthworm galleries, burrow walls and middens by

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https://doi.org/10.1016/j.ejsobi.2023.103474
Received 2 September 2022; Received in revised form 13 January 2023; Accepted 10 February 2023
Available online 21 February 2023

enhancing microbial enzyme activities compared to bulk soil [7,8,11,12]. [12] specifically found increase in enzymatic activities of dehydrogenase, protease, β-glucosidase and alkaline phosphomonoesterase in the burrow walls compared to bulk soil. Also, the microbial enzymatic activities of burrow walls were found to change from activities of bulk soil to enzymes with lower affinity to holo-cellulose and *N*-acetylglucosamine and higher affinity to proteins [13]. Generally, earthworms have proven effects on their immediate surroundings in the soil, while less is known on the effects of earthworms on microorganisms in the entire soil column.

Due to the described earthworm effects, we hypothesize that earthworms increase the microbial activity and reduce prokaryotic community diversity in the entire soil system. The objective of the present study was to evaluate the effects earthworms have on the agricultural soil microbiome. This was studied in a soil model system with and without earthworms. The microbial activity was measured by an Extracellular Enzymatic Activity (EEA) assay [14,15]. The soil prokaryote community was characterized by 16S rRNA gene metabarcoding and abundance was estimated by qPCR of 16S rRNA gene.

2. Methods

2.1. Material collection

Cultivated sandy loam soil was collected from the experimental research station at Foulumgaard, Foulum, Aarhus University (55.68 N, 12.09 E) on October 18th, 2020. The soil texture was 8% clay, 11% silt, 42% coarse sand and 36% fine sand. The soil had a bulk density of 1.4 g cm^{-3}, pH 6.5, and loss of ignition of 4.4 g 100 g^{-1} dry weight. The soil was sieved with 6 mm mesh one week prior to the experiment.

The earthworms were collected from the top 30 cm of a grassland in Roskilde, Denmark (55.68 N, 12.09 E) on November 6th, 2020. Collected earthworms were acclimated to laboratory conditions by exposing them to the soil and cow dung, for two weeks in 22 L transparent polypropylene plastic boxes in the dark at 15 °C. The cow dung used to feed the earthworms was from non-medicated animals and was dried and finely ground and then rewetted by soaking in deionized water (1:3.3 dung:water ratio) prior to use as feed source [16]. Prior to the setup of the experiment, the earthworms were left on a Petri dish with wet filter paper for 24 h to empty their guts. The earthworm wet weight (ww) was then determined to ensure equal biomass of 3–3.5 g ww in each pot. The earthworms were weighed again at harvest to encounter any weight loss. The number of earthworms in each pot is shown in Table 1.

Table 1
Summary of the final composition in each pot used in the study, as well as the percentage change in biomass recorded from start of the experiment to the end after 15 days.

Replicate	Biomass change (initial ww: 3–3.5 g)	Earthworm species (number of individuals)
1	−2.9%	*Apporectodea tuberculata* (4), *Apporectodea rosea* (1), *Apporectodea caliginosa* (2), *Apporectodea longa* (1), unidentified (2)
2	4.8%	*Apporectodea tuberculata* (2), *Apporectodea rosea* (1), *Apporectodea caliginosa* (4)
3	6.5%	*Apporectodea longa* (2), unidentified (1)

2.2. Experimental design

The experimental design employed soil with earthworms and control soil without earthworms, each group replicated three times. The experiment was carried out in a total of nine 1.1 L white plastic pots (height 14.7 cm, diameter 12.4 cm) each containing 500 g dry weight soil, covered with black plastic bags with air holes. Soil moisture was kept at 25–30% by adjusting the weight of individual pots with deionized water weekly. The rewetted cow dung suspension was applied to all pots (22.5 g pot^{-1}) on the surface of the soil at the start of experiment. The pots were incubated for 15 days in the dark at 15 °C as described by Ref. [17].

The pots were destructively sampled after 0 and 15 days. At day 0, only the soils without earthworms were sampled and used as a control. By sampling at day 15, all earthworms from each pot were removed for taxonomic identification based on DNA sequencing. The tip of each earthworm tail was cut and fixed in 96% ethanol and stored at −20 °C until DNA extraction. The soil in each pot was carefully hand-mixed and a subset was sieved through a 2 mm sieve and 5 g were transferred to 50 mL Falcon tubes for the EEA assay, which was done on the day of sampling. For DNA analyses of the microbial communities, a subset of the mixed soil was stored in two 50 mL Falcon tubes per pot at −80 °C.

2.3. Earthworm identification

Earthworms were morphologically identified in the field to genus level (*Aporrectodea* spp.) and then to species level after the experiment via sequencing the mitochondrial 16S rRNA gene. Earthworm DNA was obtained from ethanol fixed tail tips by adding 100 μL of PCR-grade water and heating at 95 °C for 15 min. The resulting liquid was used as template DNA for PCR amplification with ewD/ewE primers [18,19] targeting a ~70 bp long variable region of mitochondrial 16S rRNA gene. The PCR products were subjected to Sanger sequencing (Macrogen, Amsterdam, Netherlands) and the obtained sequence were aligned against the NCBI using BLAST database to identify earthworm at species level.

2.4. Extracellular enzyme activity assay

The activity of seven extracellular enzymes involved in soil carbon, nitrogen and phosphorus transformations (Table 2) was tested as described by Ref. [15]. The assay is based on addition of synthetic substrates bound to a fluorogenic moiety which liberates fluorescence once cleaved by the enzymes. The chemical compound 4-Methylumbelliferone (MUF) was responsible for the fluorescence in the assay.

In brief, the extracellular enzymes were extracted from soil by sonication and mixing followed by centrifugation. Aliquots (200 μL) of supernatant were transferred to 96-well black polysorp microplates

Table 2
Substrates used in the EEA analysis, their corresponding enzymes with enzyme commission numbers (EC Number), as well as associated biogeochemical cycles.

Substrates	Corresponding enzyme	Biogeochemical cycle	EC number
MUF-α-D-glucoside	α-glucosidase	Carbon	EC 3.2.1.20
MUF-β-d-glucopyranoside	β-glucosidase/cellobiase	Carbon	EC 3.2.1.21
MUF-β-D-cellobioside	Cellobiosidase	Carbon	EC 3.2.1.91
MUF-β-d-xylopyranoside	1,4-β-xylosidase/xylanase	Carbon	EC 3.2.1.37
MUF-β-D-lactoside	Endo-1,4-β-glucanase	Carbon	EC 3.2.1.4
MUF-*N*-acetyl-β-d-glucosaminide	Chitinase/*N*-acetyl-β-d-glucosaminidase	Nitrogen, carbon	EC 3.2.1.14 EC 3.2.1.52
MUF-phosphate	Phosphomonoesterase	Phosphorus	EC 3.1.3.1 EC 3.1.3.2

(Thermo Scientific), along with 10 μL 0.3 M MOPS buffer and 40 μL of relevant MUF-substrates (according to Ref. [15] and all provided by Sigma-Aldrich, Denmark), resulting in 50 μM concentration in the wells. The microplates were immediately placed in Chamaeleon Multilabel Microplate Reader (Hidex OY, Finland) and the fluorescence was measured at 10 min intervals for 80 min at 30 °C, at wavelengths of 355 nm excitation and 460 nm emission, with automated shaking in between the measurements. Enzyme activities were calculated from linear regression analysis of the standard curve for each soil sample and calculated for each time point, except the first 10 min which were omitted from the analysis. The activity was expressed in nmMUF g^{-1} dry soil h^{-1}. All samples were analyzed in triplicates.

At day 0, one control replicate of cellobiosidase and of endo-β-glucanase did not show linear increase during the 80 min measurements and the data were not included. For another replicate of control day 0 of endo-β-glucanase, we used values obtained from ten times diluted MUF-standard in standard curve calculations. Furthermore, values for MUF-standard of one replicate of day 15 control were erroneous and replaced by average values of the other two replicates.

2.5. DNA extractions

Prior to DNA extraction the soil was lyophilized for 72 h at −54 °C using a freeze drier (Scanlaf Model Coolsafe 55, Lynge, Denmark) and homogenized using a bead mill homogenizer (Bead Ruptor Elite, Omni International, USA) by adding fifteen 2.4 mm metal beads (Metal Bead Media, Omni International, USA) to each 50 ml Falcon tube containing 40 g of soil sample and running for three cycles of 30 s at 4 m s^{-1} speed. DNA was extracted from 0.25 g of soil using DNeasy PowerLyzer PowerSoil DNA Isolation Kit (Qiagen, Denmark) following the manufacturer's protocol. The DNA concentration in each sample was measured using Qubit 4.0 fluorometer (Invitrogen, Oregon, USA). The DNA was stored at −20 °C until used for MiSeq library preparation and qPCR.

2.6. Quantitative PCR

qPCR targeting the 16S rRNA gene was performed in 20 μL reactions containing 1 μL DNA template, 12.2 μL PCR-grade water, 4 μL 5x HOT FIREPol® Master mix (Solis Biodyne), and 0.4 μL of each 10 μM 341F (5′-CCTAYGGGRBGCASCA-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) primers [20]. qPCR conditions were as follows: 95 °C for 12 min followed by 40 cycles of 95 °C for 15 s, 56 °C for 30 s, 72 °C for 30 s and finally 72 °C for 3 min followed by a melt curve created by increasing the temperature in 0.5 °C increments every 5 s from 72 to 95 °C. The 16S rRNA gene standard curves were prepared from DNA extracts of *Escherichia coli* K-12 [21] with 1.97×10^8 16S rRNA gene copies per μL in the undiluted standard and six 10 times dilutions made from this. 16S rRNA gene copy numbers were calculated using Bio-Rad CFX manager 3.1 (Bio-Rad, Hercules, USA).

2.7. Library preparation and sequencing

The microbial community composition of the soil was determined by amplicon sequencing of the V3–V4 region of 16S rRNA gene through a two-step PCR-amplification protocol. The 16S rDNA region was amplified (in two technical replicates) using the 341F/806R primers with Illumina Nextera overhang adapters. The amplification mixture of 25 μL for each reaction contained 5 μL 5x PCRBIO HiFi Buffer, 0.5 U PCRBIO HiFi polymerase (PCR Biosystems Inc., United Kingdom), 0.5 μL 10 μM forward and reverse primers, 0.5 μL bovine serum albumin and 3 μL of extracted DNA template (21.6–90.0 ng DNA). PCR reactions were conducted on a SimpliAmp Thermal Cycler (Applied Bio-systems, Foster City, California, US), and cycling program was as follows: 95 °C for 2 min, 33 cycles of 95 °C for 15 s, 55 °C for 15 s, 68 °C for 40 s, and the final elongation at 68 °C for 4 min after which samples were held at 4 °C. The PCR products from two technical replicates were pooled and a second PCR was performed to attach indexes and sequencing adaptors. The 25 μL amplification mixture for the second PCR included 5 μL 5x PCRBIO HiFi Buffer, 2 μL 10 μM of each index primer, 0.5 U PCRBIO HiFi polymerase and 4 μL of the first PCR pooled product as a template. The amplification cycle was as follows: 98 °C for 1 min, 13 cycles of 98 °C for 10 s, 55 °C for 20 s, 68 °C for 20 s, and the final elongation at 68 °C for 5 min. The PCR products were then purified using 20 μL HighPrep™ magnetic beads (MagBio Genomics Inc. Gaithersburg, Maryland, US), according to manufacturer's instructions, and eluted in 27 μL TE buffer. After that, the amplicon size was confirmed on 1.5% agarose gel using SYBR® Safe DNA loading dye. Finally, 30 ng DNA of each sample were pooled. The pooled library was run on 1% agarose gel, and the band of expected size was extracted using QIAquick Gel Extraction Kit (Qiagen) and finally quantified on Qubit. Before sequencing, the library was also checked on 4150 TapeStation (Agilent Technologies, Waldbronn, Germany) using D1000 reagents, as well as by qPCR using NGSBIO Library Quant Kit for Illumina® Separate-ROX (PCR Biosystems Ltd.). The final library was sequenced on an Illumina MiSeq using the V3 kit (Illumina Inc. San Diego, California, US) resulting in 2 × 300 bp reads. To ensure enough DNA reads, the sequencing was run a second time using the same protocol and two datasets were merged using *cat* function in bash. We pooled approx. 140 ng DNA per sample, and then precipitated DNA in 21 μL 3 M sodium acetate, followed by washing two times with 70% ethanol. The rest of the procedure was repeated as described above, except that the final library was sequenced using the V2 kit, resulting in 2 × 250 bp reads. Sequences were deposited under SRA accession number PRJNA875885.

2.8. Bioinformatics

The demultiplexed DNA reads obtained from two Illumina MiSeq runs were analyzed as described earlier [22]. Forward and reverse primers were trimmed using *cut adapt* [23]. Reads were filtered, denoised, merged, chimera checked, dereplicated and clustered at 97% similarity using *vsearch*. Taxonomy assignment of representative reads was carried out using *vsearch* [24] plugin in QIIME2 [25] via classify-consensus against SILVA database v. 132 [26]. Resulting taxonomy and operational taxonomic units (OTU) tables were exported into R for further processing.

2.9. Statistics

Data obtained from EEA and qPCR were analyzed in R v. 4.0.3 [27]. Then the three treatments (control day 0, control day 15 and earthworms day 15) were subjected to analysis of variance (ANOVA) using aov function in R stats package, followed by a post hoc Tukey HSD test. Each dataset was checked for normal distribution by analyzing ANOVA model residuals using the Shapiro-Wilk test, by visually inspecting the Q-Q plot and by Levene's test for equality of variances. Data visualization was carried out using *ggplot2* v. 3.3.3 [28]. Principal components analysis (PCA) plot for all seven enzymes was made using *prcomp* function. Variance partitioning and significance for experimental factors were tested using PERMANOVA (R package *vegan*, adonis function) with 999 permutations.

Diversity-based analyses were done using the *vegan* package v. 2.5–7 [29] and the *phyloseq* package v. 1.34 [30]. Package *ggplot2* v. 3.3.3 [28] was used for data visualization. OTUs represented in less than three samples were removed. Alpha diversity was estimated using the observed richness (number of OTUs) and Shannon diversity measures on data that was 100 times rarefied to the lowest sequencing depth and the mean diversity estimates were used. Significant differences among treatments within diversity estimates were determined using the ANOVA test followed by a Tukey HSD test. Bray-Curtis distance matrices were used for beta diversity analysis at OTU level using data transformed to relative abundance. Variance partitioning and significance for experimental factors were tested using PERMANOVA (R package *vegan*,

adonis function) with 999 permutations. Dissimilarity between samples based on Bray-Curtis distances was visualized using Principal Coordinate Analysis (PCoA). Differentially abundant OTUs (when p-value <0.05 using Wald test with Benjamini-Hochberg adjustment) in the treatments with and without earthworms were identified using *DESeq2* package [31].

The Spearman's rank correlations between the extracellular enzyme activities and the relative abundance of prokaryotic OTUs of the soil microbiome were calculated using rcorr function from the *Hmisc* package in R. Correlations were calculated on OTUs with at least 10 reads across samples. Only the significant correlations ($p < 0.01$) with Spearman's rank correlation coefficient >0.6 or < −0.6 were reported.

3. Results

3.1. Earthworm identification and mortality

Earthworms were morphologically identified as *Aporrectodea* spp. while species identification of all individuals was not possible due to many juveniles. The identification was supported by DNA-based identification further identifying the worms to four different species while three remained unidentified (Table 1). The earthworm biomass had either decreased (replicate 1) or increased (replicate 2 and 3) by the end of the experiment (Table 1), and one earthworm (in replicate 2) was found dead.

3.2. Enzymatic activity

The soil extracellular enzymatic activities ranged from on average 1.3 nmMUF g^{-1} dry soil h^{-1} for endo-β-glucanase, to 544.5 nmMUF g^{-1} dry soil h^{-1} for phosphomonoesterase (Table 3). Significant differences between control at day 0 and 15 were found only for chitinase ($p = 0.03$). In contrast, the presence of earthworms increased enzymatic activity in the mixed soil compared to day 0 of α-glucosidase ($p = 0.02$), β-glucosidase ($p = 0.035$), chitinase ($p = 0.005$) and endo-β-glucanase ($p = 0.008$) as found by one-way ANOVA followed by Tukey HSD. However, after 15 days significant difference between earthworm and control was only found for endo-β-glucanase ($p = 0.04$). Principal Component Analysis (PCA) plot based on seven enzyme activities revealed significant clustering based on presence/absence of earthworms and on sampling time (Fig. 1a.) (PERMANOVA Adonis, $p = 0.049$). Hence, both incubation time and presence of earthworms significantly affected the soil extracellular enzymatic activities.

3.3. Prokaryote abundance

Total number of soil prokaryotes was reflected in the 16S rRNA gene copy numbers found by qPCR as follows as 16S rRNA genes g^{-1} dry soil: on average 5.3×10^9 (standard deviation (sd) $\pm 1.2 \times 10^9$) in soil with earthworms at day 15; 3.9×10^9 (sd $\pm 6.1 \times 10^8$) in soil without earthworms at day 15, and 3×10^9 (sd $\pm 1.8 \times 10^9$) at day 0. No significant differences were found among the treatments.

Table 3
Activity (nmMUF g^{-1} dry soil h^{-1}) of seven different enzymes from control soils and with earthworms at two sampling times. Data are presented as means with standard deviation (n = 3, except at T_0 of cellobiosidase and endo-β-glucanase, where n = 2). Significant difference (α = 0.05) between groups of each of the 7 enzymes is indicated by different letters.

	Control Day 0 (nmMUF g^{-1} dry soil h^{-1})	Control Day 15 (nmMUF g^{-1} dry soil h^{-1})	Earthworms Day 15 (nmMUF g^{-1} dry soil h^{-1})
α-glucosidase	3.8 ± 1.2 b	9.4 ± 3.1 ab	19.7 ± 8.1 a
β-glucosidase	32 ± 16.5 b	71 ± 11.4 ab	91.3 ± 31.6 a
β-xylosidase	3.8 ± 3.1 a	12.1 ± 2.7 a	39.1 ± 31.4 a
Cellobiosidase	2.4 ± 0.4 a	7.2 ± 4.1 a	25 ± 20 a
Chitinase	15.6 ± 9.5 b	57.3 ± 10.6 a	75.7 ± 21 a
Endo-β-glucanase	1.3 ± 0.5 b	2.3 ± 1.3 b	6.1 ± 1.8 a
Phosphomonoesterase	295.1 ± 128.3 a	544.5 ± 65.4 a	437.4 ± 91.3 a

3.4. Prokaryotic community structure

Data characteristics: After quality control, taxonomy assignment, and removing singletons, we obtained 98.8 thousand prokaryote reads clustered into 2166 OTUs. Minimum reads per sample were 2,861, maximum 17,059, and the median was 12,701. The presence of earthworms significantly decreased the alpha diversity of the prokaryotic community as measured by observed richness. Shannon diversity index, on the other hand, was not affected ($p = 0.06$) (Table 4).

Prokaryotic community structure clustered based on presence or absence of earthworms in a PCoA analysis (Fig. 1b). PERMANOVA found significant differences between prokaryotic communities in the three treatments ($p = 0.008$, $r^2 = 0.53$).

The 16S rRNA gene amplicon sequencing showed that prokaryotic communities were dominated by *Firmicutes* (relative abundance 34% on average among treatments), *Proteobacteria* (21%) and *Actinobacteria* (19%) (Fig. 2). Earthworm treatment showed higher relative abundance of *Bacteroidetes* and lower relative abundance of *Thaumarchaeota* compared to control treatments.

Differential abundance analysis using DESeq2 identified significantly differentially abundant OTUs between soils with and without earthworms after 15 days (Fig. 3). In total, 37 OTUs were found to be differentially abundant, with 27 being enriched in soils with earthworms and the remaining ten in soils without earthworms. Earthworms increased relative abundance of bacterial taxa within *Bacteroidetes* and *Proteobacteria* phyla, whereas those enriched in soil without earthworms mainly belonged to the archaeal phylum *Thaumarchaeota* (Fig. 3).

The extracellular enzyme activity showed strong correlations with the relative abundance of several bacterial genera of the soil microbiome for six enzymatic activities, showing both positive and negative correlations. Specifically, the *Bacillus* and *Sporosarcina* of *Firmicutes*, the *Smaragdicoccus* of *Actinobacteria*, the *Hyphomicrobium* and an uncultured member of *Proteobacteria* and the uncultured *Chloroflexi* negatively correlated with enzymatic activities. In contrast, three members of bacteria within *Firmicutes*, nine *Proteobacteria* and three *Bacteroidetes* all showed positive correlation with enzymatic activities (Fig. 4). As there was no significant change in bacterial abundance over time, neither in the control nor in earthworm treatments, the relative abundance of bacterial OTUs is directly related to their actual abundance.

4. Discussion

Here, we demonstrate earthworms' effect on extracellular enzyme activities and the prokaryotic community structure in agricultural soil. The most dominating earthworm genus in the field was *Apporectodea*, and hence this genus was used for the experiment. However, this genus consists of different ecotypes, and only after the genetic identification of the species this difference was determined. Alternative approach to avoid this mix of *Apporectodea* species, could be to use clonal cultures, however with less ecological relevance, or only adult individuals sampled in the field.

The experimental setup was designed to study effects on the entire soil as affected by the earthworm activities of burrowing, soil mixing, introducing and comminuting organic matter etc. in order to get an overall assessment of the earthworm effects. During the incubation of earthworms in soil the earthworm biomass showed a weight loss/gain less than 30% which is considered acceptable according to Ref. [32].

We observed that the activity of selected extracellular enzymes increased significantly with incubation time, and the prokaryotic community structure was altered in the presence of earthworms after 15 days. *Aporrectodea longa*, the dominating earthworm in replicate 3 and with one worm in replicate 1, is an anecic earthworm and belong to a

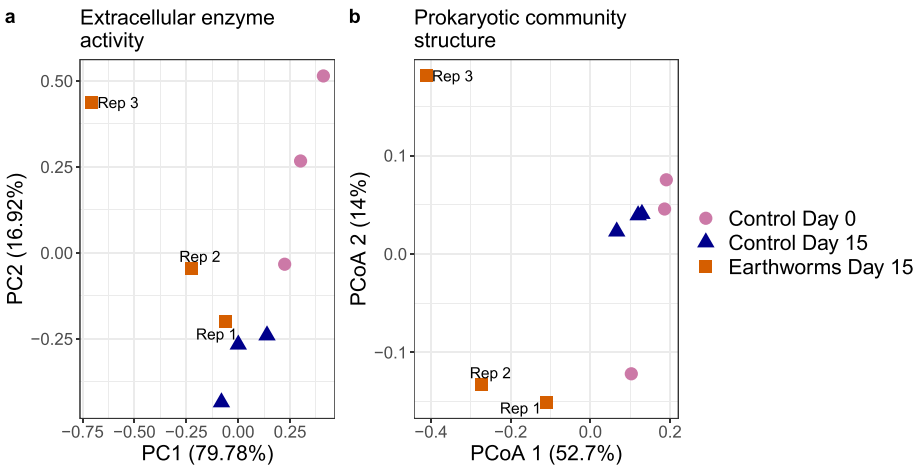

Fig. 1. a) PCA plot with activities of the seven enzymes of the EEA assay in control soils after 0 and 15 days and in soil with earthworms after 15 days. b) PCoA plot of prokaryotic community structure based on OTUs in control soils after 0 and 15 days and in soil with earthworms after 15 days. Replicate number of samples with earthworms is indicated.

Table 4
Alpha diversities estimated using observed OTU richness and Shannon diversity index in soil with earthworms and control soils at two time points (day 0 and 15). Data are presented as means with standard deviation (n = 3). Significant difference (α = 0.05) between groups is indicated by bold letters.

	Control day 0	Control day 15	Earthworms day 15
Observed OTU richness	574.9 ± 8.1 **a**	623.9 ± 20.6 **a**	518.1 ± 27.2 **b**
Shannon	5.1 ± 0.1 **a**	5.3 ± 0.0 **a**	5.3 ± 0.1 **a**

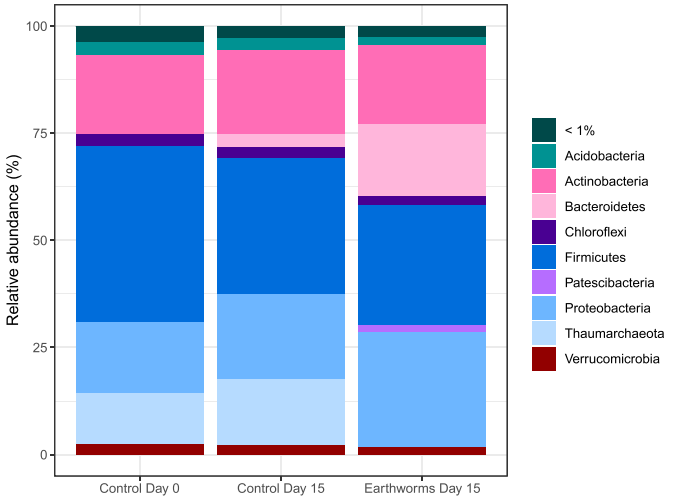

Fig. 2. Relative abundance of prokaryote communities at phylum level in soils with earthworms and without earthworms (control) at days 0 and 15. Phyla with relative abundance of <1% in each treatment were grouped together.

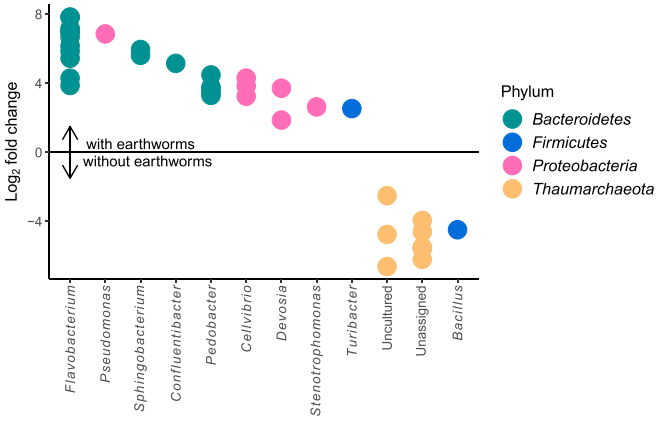

Fig. 3. Significantly differentially abundant OTUs (shown at genus level) and their $\log_2$ fold change after 15 days. OTUs not assigned at genus level are grouped together as "Unassigned". Each genus is also grouped to a phylum level by color.

different ecotype than the other *Aporrectodea* spp being endogeic earthworms. Such difference likely explains the distinct behavior of replicate 3 in extracellular enzyme activity and prokaryotic community structure, and add information about the ecological mechanisms that take place in the pots as also found by Ref. [11].

The EEA measurements showed that incubation for 15 days generally increased microbial activity. The EEA assay was applied for seven different hydrolytic enzymes extracted from soils containing earthworms and control soil without earthworms. Activity of chitinase was significantly higher after 15 days in both the control and earthworm treatments compared to day 0. Chitinase is an important enzyme in degradation of constituent of fungal hyphae chitin [33], which might have been present in cow dung [34]. The remaining three enzymes with significant increased activity in earthworm treatments (α-glucosidase, β-glucosidase, endo-β-glucanase) compared to day 0, were cellulases, which is a group of hydrolytic enzymes that microorganisms produce to decompose polysaccharides.

Of these enzymes only endo-β-glucanase was significantly higher in the earthworm treatment after 15 days compared to control after 15 days. Hence, the cow dung stimulated the chitinase activity and earthworms further stimulated the activity of cellulases. Specifically, endo-β-glucanase is responsible for cleaving internal β-1,4-glucan units and hydrolyze polysaccharides [35,36] and is also involved in many aspects of plant growth, especially during differentiation of xylem cells. Fungi have been suggested as a predominant source for this specific enzyme [37,38]. Hence, this suggests that earthworms stimulate fungal activity. The finding of earthworm inducing increased enzymatic activities has also been reported by Ref. [12] who found differences between earthworm burrows and bulk soil. [11] also found differences in the stimulation of enzymatic activities to depend on ecotype and that FDAse activity was higher in burrows and acid phosphatase higher in middens. We only found statistical stimulating earthworm effects on the activity of endo-β-glucanase, which we believe is due to a dilution effect of the

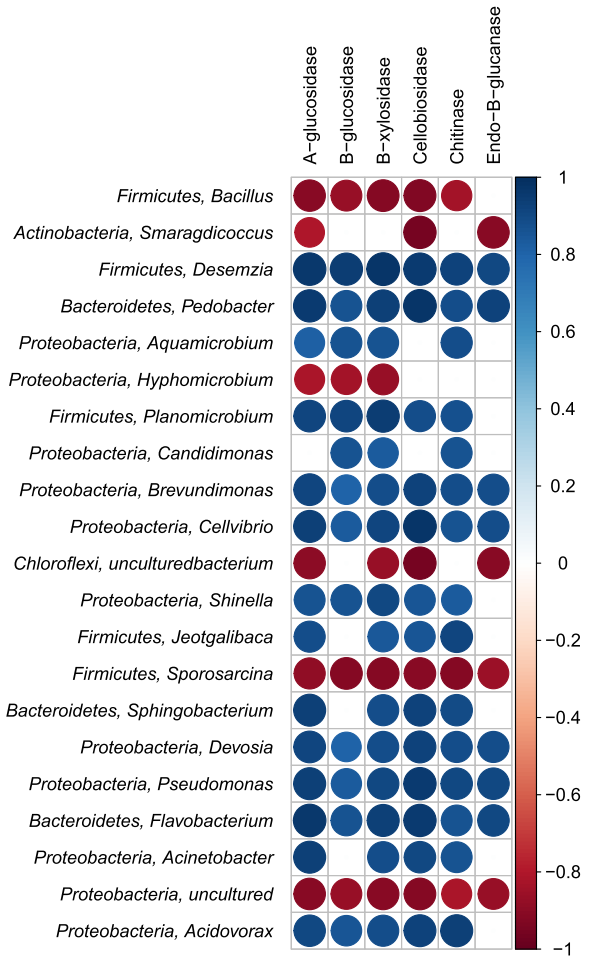

Fig. 4. Spearman's rank correlation analysis between relative abundance of bacterial genera and extracellular enzymes. Empty cells indicate non-significant correlation ($\alpha = 0.01$). The vertical color bar legend indicates correlation coefficient (blue: positive; red: negative).

earthworm impact on the total mixed soil.

Earthworms had no apparent effects on soil prokaryote abundance in our soil experiment. There is little doubt in the literature that earthworms influence microbial communities, especially in distinctive microniches as burrow walls and casts where several references have found earthworms to increase microbial abundance, activity and diversity depending on the food source [3,5–8,13]. However, contrasting results exist whether the influence is positive, neutral, or negative, as summarized in Ref. [4]. The consensus presented in this review is that earthworms have limited effects when the feed added is rich in organic matter. This could also be the case in our study, as cow dung supplements the soil with nutrients and readily available carbon sources [39]. Lastly, the lack of significant effect on overall prokaryote abundance could be masked by the bulk soil to earthworm affected soil relation and also be explained by changes in relative abundance of certain prokaryotic taxa, as seen in Fig. 3.

As expected, earthworms decreased the prokaryotic alpha diversity in soil with earthworms (Table 4). As earthworms change the soil properties, certain microorganisms are favored and thus selected for, which changes microbial population dynamics [6,8,40,41]. We found that addition of earthworms affected the microbial community structure in the soil, and significant associations were found with parts of soil microbiota. In particular, 19 *Bacteroidetes* and seven *Proteobacteria* OTUs were found to be enriched in soil with the addition of earthworms, while *Thaumarchaeota* (nine OTUs) were enriched in soil without earthworms (Fig. 3).

Flavobacterium, *Pedobacter, Confluentibacter* and *Sphingobacterium,* four genera of phylum *Bacteroidetes,* responded positively to the presence of earthworms. Their positive association with the earthworms is in agreement with earlier studies [42–44], except for *Confluentibacter*, which has not been reported to be associated with earthworms in previous studies. Indeed [42], suggested that the increase in *Flavobacteriaceae* could be attributed to an increase in nitrogen supplied by earthworm mucus, which would activate fast growing *Proteobacteria* members, such as *Flavobacteriaceae*. *Pseudomonas*, *Cellvibrio, Stenotrophomonas* and *Devosia*. Likewise other studies also point towards proteobacteria, especially *Pseudomonas,* to be associated with earthworms [40,45,46]. On the other hand, the taxa that were enriched in soil without earthworms mainly belong to *Thaumarchaeota*, a phylum consisting of ammonia oxidizing archaea. [47] showed that ammonia oxidizing archaea were promoted in vermicompost systems but the effects of earthworms on *Thaumarchaeota* seem to be understudied. The opposing results could be due to several different factors originating from different experimental systems (soil with cow dung *versus* fruit and vegetable waste compost), different techniques (amplicon sequencing of 16S rRNA gene *versus* qPCR of *amoA*), as well as different earthworms studied (mainly *Aporrectodea* spp. *versus Eisenia fetida*).

We found strong correlations with differential abundance analysis between enzymatic activities (all except phosphomonoesterase) and relative abundances of certain bacterial OTUs (Fig. 4). This could indicate that these bacteria were responsible for the enzymatic activities observed. *Pedobacter, Cellvibrio, Devosia, Pseudomonas* and *Flavobacterium* showed strong correlations with the activities of all six enzymes. We further checked whether these overlapping taxa could contain genes coding for the respective enzymes they were found to be associated with, based on Kegg database [48] and other published literature [58]. Bacteria from the genus *Cellvibrio* are usually aerobic and considered saprophytic because they can degrade cellulose, dextran, xylan and chitin [58]. Various *Cellvibrio* strains were found to have genes coding for all six associated enzymes. *Pedobacter* and *Flavobacterium* are anaerobic bacteria, although aerobic growth has also been observed in a few *Flavobacterium* species. Many *Pedobacter* and *Flavobacterium* can produce numerous different extracellular enzymes for degradation of biopolymers, including the correlated enzymes, except for cellobiosidase [49,50]. *Pseudomonas* genus is one of the most diverse bacterial groups and is known for its metabolic versatility [51], although it likely does not have cellobiosidase activity. *Devosia* is another diverse and ubiquitous genus, often found in contaminated environments [52], although not yet known to have cellobiosidase and chitinase genes. Furthermore, *Sphingobacterium* has been positively correlated with α-glucosidase, cellobiosidase, β-xylosidase and chitinase, and members of this genus appear to be capable of synthesizing these enzymes [53]. So, all genera that were enriched in the presence of earthworms are capable of hydrolyzing enzymes that showed increased activity in the presence of earthworms in the EEA assay. An exception is cellobiosidase and we hypothesize that fungi can be a predominant source of this enzyme; however, fungi were not investigated in our study.

In conclusion, our study confirms the hypothesis that earthworms indeed modified the soil microbiome and extracellular enzyme activity across the entire soil. Specifically, earthworms decreased prokaryote alpha diversity by favoring certain microbial taxa. Moreover, earthworms have a positive effect on some of the enzymatic processes in soil, which could be related to enrichment of bacteria producing such enzymes.

Author statements

ZB and MAL designed the experiment, conducted laboratory work and wrote the original draft. LEJ supervised and conducted Illumina sequencing. NBH and PHK supervised experimental setup and contributed in earthworm collection and culturing. ZB, MAL and RS analyzed the data. RS, BWH and AW supervised the study. AW secured the

funding and led the study. All authors read, revised and approved the manuscript.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgments

The study was supported by the Danish Climate Research Program, project KLIMINI grant no. 33010-NIFA-19-726, the eDNA Center at Technical Sciences, Aarhus University, and Aarhus University Interdisciplinary Centre for Climate Change (iClimate, Aarhus University). We would like to express appreciation to laboratory technicians Tanja Begovic and Tina Thane for their assistance.

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