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**Effect of an organophosphorus insecticide, soil texture and earthworm species on the turnover of soil, gut and cast microbiota during the earthworm's gut transit.**

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## **Abstract**

The growing usage of plant protection products in agroecosystems questions their unintentional deleterious effects on non-target organisms such as earthworms and their related ecological function. The aim of this study was to investigate the turnover of soil microbiota during the gut transit of two endogeic earthworms (*Aporrectodea caliginosa* and *Allolobophora chlorotica*) and how it is possibly affected by the organophosphorus insecticide ethyl-parathion (Pt). We measured prokaryotic and micro-eukaryotic community composition and abundances using two different soil types in the continuum of bulk soil, gut and casts samples collected during and after one week exposure to Pt. The presence of earthworm and Pt had limited effects on the soil microbiota, although Pt altered temporarily soil microbial abundances in the presence of earthworms, not in their absence. The earthworms' gut harbored a remarkably low prokaryotic diversity, dominated by two molecular Operational Taxonomic Units (mOTUs) affiliated to *Rhodococcus* and *Pseudarthrobacter*. Contrastingly, gut micro-eukaryotic communities were maintained at diversity levels similar to that of the soil, yet Pt augmented their diversity and changed their composition. Fresh casts collected out of the soil revealed evident variation of abundance, diversity and community composition according to the soil origin and the earthworm species. In particular, following gut transit casts were enriched with Bacteroidia and  $\gamma$ -Proteobacteria while depleted of Acidobacteria. Exposure to Pt increased the diversity and relative abundance of depleted mOTUs, which paves the way for future investigations on the role of Pt in rising microbial consumption due to an over-excitation of gastrointestinal motility.

## **Keywords**

*Aporrectodea caliginosa*; *Allolobophora chlorotica*; parathion; earthworm's gut microbiome; prokaryote and micro-eukaryote microbiota

## 1. Introduction

The role of earthworms in ecosystems has received much attention since they are a dominant component of the soil fauna and considered key bioindicator of the soil quality (Jones et al., 1994; Edwards, 2004). By burrowing and ingesting the soil they have determining effects on the overall soil structure, the organic matter turnover and its transport into the soil column, providing available nutrients and aeration for other soil organisms (Lavelle, 1988; Edwards and Bohlen, 1996; Jégou et al., 2000). It is assumed that over a year 4-10% of soil is passing through the total population of earthworms' gut (Edwards and Bohlen, 1996) and during the earthworms' gut transit, the abundance, the composition and related enzymatic activities of soil microbial communities change (Aira et al., 2015; Dempsey et al., 2011; Tao et al., 2009; Lipiec et al., 2016).

The earthworm's gut is considered as an anaerobic medium prospering in an aerobic environment, with stable moisture conditions and nutrient content (Horn et al., 2005; Drake and Horn, 2007). It acts as a biological filter where some ingested microorganisms could be either digested or stimulated (Wüst et al., 2011; Zeibich et al., 2019; Aira et al., 2015; Aira et al., 2022a). Therefore, the modifications of the microbial communities along the gut transit contribute to differentiate microbial composition of dejected casts compared to that of the ingested soil.

Whenever they can survive and proliferate during gut transit, ingested microorganisms provide exoenzymes that enhance the degradation of organic matter into the gut and help the earthworm's assimilation of nutrients (Medina-Sauza et al., 2019). Environmental conditions of the earthworm's gut were shown to favor fermentation processes mediated by anaerobic prokaryotic groups able to digest stable organic matter such as cellulose (Edwards and Fletcher, 1988; Wüst et al., 2011). In addition, the production of mucus that provides water, readily available carbon sources and neutral pH is partly responsible for the mutualistic relationship with the gut microbiota (Trigo and Lavelle, 1995; Brown et al., 2000).

Conversely, the gut mucus was shown to be detrimental to some prokaryotic and fungal isolates as it induces the lysis of their cell membranes, thus contributing to the host nutrition, the maintenance of a stable gut microbiota and the alteration of the soil microbial communities (Byzov et al., 2007).

The variations of abundance and composition of earthworms' gut microbiota during the digestive transit was shown to differ according to the earthworm ecological category and the food intake (Singleton et al., 2003; Brito-Vega and Espinosa-Victoria, 2009; Thakuria et al., 2010). Besides variation of composition and abundance of the gut microbiome, the possible

existence of particular microbial groups inhabiting the earthworm's gut or having established symbiosis with their host have been suggested (Singleton et al., 2003; Aira et al., 2022; Valle-Molinares et al., 2007). All of which emphasize the interest to study the composition, structure and diversity of soil prokaryotic communities and their dynamic during the earthworm gut transit (Aira et al., 2022; Bi et al., 2021).

Considered as non-target organisms, earthworms play an important role in terrestrial ecotoxicology and have been used as bioindicators to study pesticides and chemical effects (Scott-Fordsmand and Weeks, 2000; Sanchez-Hernandez, 2006; Pelosi et al., 2014). Toxicity assessments using earthworms as models have mainly focused on abundance (Amossé et al., 2018), behavior (Capowiez et al., 2010; Martinez Morcillo et al., 2013), reproduction (Yasmin and D'Souza, 2010), enzymatic biomarkers (Jouni et al., 2018; 2021) and cellular processes (Dutta and Dutta, 2016). Although the gut microbiota has been shown to contribute to host gut, metabolic, and immune homeostasis (Devkota and Chang, 2013), the interest in the role of the gut microbiota in pesticide-induced toxicity in non-target organisms is limited to insects and vertebrates (Giambo et al., 2021; Siddiqui et al., 2022). The few studies that have considered the role of earthworm gut microbiota in the response to pesticides inputs revealed that a gut prokaryotic isolate could degrade Endosulfan in vitro (Verma et al., 2006), that earthworms may filter out bacterial degraders of the herbicide 4-chloro-2-methylphenoxyacetic acid (Liu et al., 2013) or that some gut microbial groups were particularly sensitive to various pesticides (Ma et al., 2017; Astaykina et al., 2022), calling for standard consideration of the earthworm gut microbiota in pesticide risk assessment protocols.

The objective of this study was to investigate possible effects of the organophosphate insecticide ethyl-parathion (Pt) on the gut microbiota of two endogeic earthworm species (*Aporrectodea caliginosa* and *Allolobophora chlorotica*) exhibiting contrasted sensitivity to ethyl-parathion. We used an amplicon-sequencing approach to decipher prokaryotic and micro-eukaryotic communities present in two soils showing different physicochemical properties, the earthworms' intestine and their dejected casts. We measured changes of microbial abundance and composition in each compartment as a function of exposure time, earthworm species and soil type. We assess the microbial turnover of soil microbiota during gut transit by comparing the ingested soil to the dejected casts and we paid a particular

attention to fractions of the soil microbial diversity that were upheld in casts or depleted from soil during digestive transit.

## 2. Materials and methods

### 2.1. Sample collection and experimental setting

Soils and earthworms were collected from two different apple orchards that had received no pesticide treatments for the last 15 years, located at 10 km distance from each other in the city of Montfavet, near Avignon (southeastern France). Both soils used were Luvisols (USDA). The first orchard is characterized by a silt loamy soil (Soil K: 23.4% clay, 57% silt, 19.6% sand, 28.3g.kg<sup>-1</sup> organic matter, pH 8.3) and the earthworm community there is dominated (in abundance) by the species *Allolobophora chlorotica*. The second orchard is characterized by a silt-clay soil (Soil G: 38.3% clay, 42.2% fine silt, 19.5% sand, 34g.kg<sup>-1</sup> organic matter, pH 8.5) and is dominated by the species *Aporrectodea caliginosa*. Healthy and adults earthworms from each species were collected manually from their original orchard, washed in tap water, blotted dried on filter paper and kept in the laboratory into plastic pots filled with freshly collected soil.

For cross-acclimation, each earthworm species spent 5 days in a dark cold chamber ( $12 \pm 1^\circ\text{C}$ ) either in the original or in the unusual soil. Soils were sieved at <2 mm mesh size, and their water content was adjusted to 20-21% on a weight basis with distilled water (~ 80% of the maximum water holding capacity). Wet soil samples were spiked with ethyl-parathion (parathion hereafter, Pt) solutions to obtain a final concentration of 1 mg active ingredient (a.i.) kg<sup>-1</sup> wet soil, which refers to the usual application rate and calculation of the predicted environmental concentration (Jouni et al., 2018). Control soils were prepared in the same conditions with a water solution free of parathion. The polluted (Pt) and unpolluted (control) soils were then equally distributed in plastic pots so that each contained 100 g soil.

### 2.2. Experimental design

After the acclimation period, earthworms were washed with tap water, blotted dried on filter paper and weighed. For each experiment, two soils (G and K), two soil conditions (control and with parathion), three earthworm conditions (earthworm-free (EW-free), *A. caliginosa*, *A. chlorotica*), and four replicates were used. Therefore, 48 pots were prepared (16 pots for each earthworm condition). For the pots with earthworms, two individuals were placed in each pot to obtain enough casts (total n=32 earthworms for each species) and prevent cascade death (Sheppard and Evenden, 1992). Pots were kept in a dark cold chamber ( $12 \pm 1^\circ\text{C}$ ) for the entire

experiment duration. Soils without earthworms were used as controls. After 4 days, bulk soil samples (5 g) and casts produced were gently collected from each replicate and for each condition, without touching or removing the earthworms to avoid any disturbance. After 7 days, earthworms were removed from the boxes prior to collect bulk soil and casts samples. Bulk soil and cast samples were stored in microtubes at -80°C until DNA extraction. The removed earthworms (2 earthworms per condition) were placed for 5 more days in Petri dishes containing moist filter paper, to void their gut. Fresh casts were then collected at end-point sampling corresponding to 12-days duration (d12). Earthworms' dissection was done following washing with distilled water and scarification by brief immersion in water at 50°C (Singleton et al., 2003). The guts were removed (2 earthworm's gut samples per condition) and stored at -80°C until DNA extraction.

### *2.3. DNA extraction*

DNA extraction was achieved using PowerSoil-htp 96 wells DNA Isolation Kit (MOBIO) according to the manufacturer's protocol. To which we added an initial washing step of the gut samples with the DNA extraction buffer (three times of 1-minute vortexing followed by 1-minute centrifugation) to clear remaining soil particles. Washing suspensions were gathered and processed separately from the remaining gut material. The nucleic acids concentration and quality were measured using a Nanodrop One C spectrophotometer (Thermofisher). DNA samples were stored at -20 °C until PCR amplification and sequencing.

### *2.4. Quantitative PCR procedure*

Quantitative real-time PCR (qPCR) was performed using a LightCycler 480 system (Roche Applied Science) to determine copy numbers of the prokaryotic 16S rDNA gene (Fierer et al., 2005), using primer Eub338 (5'-ACTCCTACGGGAGGCAGCAG-3') and primer Eub518 (5'-ATTACCGCGGCTGCTGG-3') and copy numbers of the universal ITS1 marker, using primer ITS1f (5'-TCCGTAGGTGAACCTGCGG-3') and primer ITS1r (5'-CGCTGCGTTCTTCATCG-3') (Fierer et al., 2005). Standard solutions of target genes were generated so to represent the naturally occurring mixture of each target gene across a random selection of 10 DNA samples (Hasset et al., 2009). Following gradient PCR tests in a Mastercycler (Eppendorf), annealing temperatures were determined at 55°C for each primer pair. PCR mixtures (20 µl) contained 200 µM dNTPs, 5% (v:v) DMSO, 1 µM of each primer and 0.25 U FlexyTaq DNA-polymerase with the corresponding 5X PCR buffer (Promega). Reaction condition had an initial denaturation step of 10 minutes at 95°C, followed by 30

cycles of amplification consisting of 30 seconds denaturation at 95°C, 30 seconds annealing at 55°C, 10 seconds extension at 72°C and a final extension step of 10 min at 72°C. Amplification products were verified by using 2% Nusieve agarose gel electrophoresis (Lonza). PCR products were mixed following extraction from agarose gel (Nucleospin Gel and PCR clean up kit, Macherey Nagel). The DNA concentration of the standard solutions was determined using a Nanodrop OneC spectrophotometer (Thermofisher) and serial decimal dilutions were used as standards. For real time quantitative PCR, the mixtures contained 10 µl of SYBR green I master mix (Roche Applied Science), 1 µM each primer (final concentration), 2 µl of DNA template and ultrapure water to fill 20 µl. Reaction condition used the same program as for preparing standard solutions but with 45 cycles amplification and duration of denaturing, annealing and extension steps was reduced to 15s. The final step was set up to determine the melting temperature of the amplified product through slow increase of temperature (0.2°C.s<sup>-1</sup>) between 50°C and 95°C. Quantification of 16SrDNA- and ITS- target genes were expressed as gene copy number per gram of fresh material (gcn.g<sup>-1</sup>).

### *2.5. Tagged amplicon sequencing*

Prokaryotic amplicons have been obtained with universal primers Bakt\_515F-Y 5'-GTGYCAGCMGCCGCGGTA-3' (Parada et al., 2016) and Bakt\_928R 5'-CCCCGYCAATTCMTTTRAGT-3' (Wang and Quian, 2009), which were designed to amplify the V4-V5 region of the bacterial and archaeal 16S rDNA genes. Primers ITS1F\_KYO2 5'-TAGAGGAAGTAAAAGTCGTAA-3' and ITS2R\_KYO2 5'-GACTACHVGGGTATCTAATCC-3', which were designed to amplify the ITS1 region lying between the 18S and 5.8S ribosomal genes and profile fungal communities (Bokulich and Mills, 2013), were used here to decipher a broader soil micro-eukaryotic community (see also the reference ITS database used in section 2.6). The primers were complemented with illumina adapters CTTTCCCTACACGACGCTCTTCCGATCT, for Bakt\_341F and ITS1F\_KYO2 and GGAGTTCAGACGTGTGCTCTTCCGATCT, for Bakt\_805R and ITS2R\_KYO2. PCR amplification was performed in a reaction mixture of 25 µl containing 200 µM dNTPs, 5% (v:v) DMSO, 1 µM of each primer and 0.5 U Pfu DNA polymerase with corresponding 10X PCR buffer (Invitrogen). Reaction condition had an initial denaturation step of 2 minutes at 95°C, followed by 35 cycles of amplification consisting of 40 seconds denaturation at 95°C, 45 seconds annealing at 60°C, 1 minute elongation at 72°C and a final elongation step of 2 minutes at 72°C. Following verification of amplicons through 2%



Nusieve agarose gel electrophoresis (Lonza) and optimization of amplification conditions when necessary, prokaryotic and micro-eukaryotic amplicons of the same sample were mixed together. Further amplicon purification, sample specific tagging and Illumina sequencing was achieved at the Plateforme Genomique Genotoul (Toulouse, France).

## 2.6. Bioinformatic analysis

Illumina reads sequences were processed following Mahé's metabarcoding analysis pipeline (<https://github.com/frederic-mahe/swarm/wiki/Fred%27s-metabarcoding-pipeline>) with some modifications. Briefly, raw paired-end Illumina reads ( $2 \times 250$  pb) were assembled to produce contigs using VSEARCH v1.10.1. (Rognes et al., 2016). "Orphan" reads with little or no overlap between pairs, or less than 32 bp in length were discarded (Mahé, 2016).

Demultiplexing of the samples based on unique nucleotide tags incorporated into raw sequence data and of the adaptor and index sequences were processed using CUTADAPT v1.9.1. Poorly aligned sequences were removed and overhangs at both ends were trimmed so that they overlap the same region. Reads were clustered with SWARM v2.1.8 (Mahé et al., 2014; Rognes et al., 2016) that regroup reads iteratively using a non-arbitrary local clustering threshold ( $d=1$ ) independently of the input order of sequences, along with a centroid sequence holding the outmost number of identical reads. Molecular Operational Taxonomic Units (mOTUs) are thus completed when no new amplicon with  $d$  or fewer differences can be assigned to the regrouped sequences. A second cluster was computed with the "fastidious" option to reduce the number of small and subgrouping mOTUs. Taxonomic assignment used the algorithm STAMPA (Sequence Taxonomic Assignment by Massive Pairwise Alignments) that allows to assign a taxa to an amplicon sequence or the last common ancestor and help delimit species (Coissac et al., 2012). The method relies on pairwise comparisons of unknown single representative sequences of each mOTU via the alignment programs VSEARCH with a reference database (Mahé, 2016). The complete small rRNA subunit reference database SILVA 132 SSU Ref updated in December 2017 was used for 16S rDNA sequences.

Reference fragments were extracted from the database using CUTADAPT so that they cover the same region as the amplicons, between forward and reverse primers. For the taxonomic assignment of ITS sequences of representative mOTUs, a local alignment was performed with BLASTN (Altschul et al., 1997) against the NCBI's non-redundant nucleotide reference database (nt that include all GenBank, EMBL, DDBJ, PDB sequences Flat File Release 220.0), allowing to add to the fungal diversity several other taxonomic divisions, considered hereafter as micro-eukaryotic diversity. All mOTUs were broadly categorized according to

known phylum characteristics and stored in mOTU abundance and taxonomic matrices (Douglas et al., 2012). To limit false positives during determination of the species list (Coissac et al., 2012), mOTUs were passed through a quality control filter that removes sequences with ambiguous nucleotides and with low quality scores (strictly greater than 0.0002). Singleton, chimeras (Deiner et al., 2017) and low abundance mOTUs (i.e. when the total number of unique sequences was strictly less than 3 or unique sequences were present in fewer than 2 samples; Moyer et al., 2014) were rejected.

### *2.7. Microbial abundance and diversity analysis*

Prokaryotic and micro-eukaryotic mOTUs' abundance matrices were analyzed with PRIMER v7 and PERMANOVA+ programs (Primer-E Ltd, Plymouth, United Kingdom; Klark and Gorley, 2015). Experimental design was used to inform sample factors and taxonomic affiliation levels as indicators of mOTUs' number. To analyze alpha-diversity of microbial communities and draw diversity profiles (Leinster and Cobbold, 2012), we used the "DIVERSE" routine to obtain the Hill numbers (Chao et al., 2014):  $H_0 = \text{mOTUs' richness}$ ,  $H_1 = \exp(-\sum p_i \times \log p_i)$  that inform on the number of typical mOTUs and  $H_2 = 1 / \sum p_i^2$  that inform on the number of dominant mOTUs ( $p_i$  is the proportion of the total abundance arising from the  $i$ th species). We used the variable indicators to provide mOTUs with their taxonomic affiliation from phylum to species levels, which allowed measuring phylum dominance as the number of mOTUs affiliated to the same phylum. To analyze beta-diversity of microbial communities, the abundance values of each mOTU were standardized by total abundance of the sample and then log-transformed ( $\log X+1$ ) before generating Bray-Curtis resemblance matrices. To visualize distance between samples, we used Principal Coordinate Ordination (PCO) plots. We used the PERMANOVA routine and Monte Carlo tests (pMC) to measure dissimilarity between community samples according to experimental conditions. To search an effect on particular taxonomic groups, we analyzed beta-diversity on selected taxonomic group in the variable indicators.

To analyze quantitative PCR data and diversity indices, we used JMP 9.0 (SAS Institute Inc.). After testing normal distribution using the Shapiro-Wilk W test, quantitative PCR values were log transformed ( $\log \text{gcn.g}^{-1}$ ) before analysis of variance. When the variances were not equal, we used the Welsh Anova test. We compared across Hill numbers diversity profiles using one-way repeated measures MANOVA between conditions.

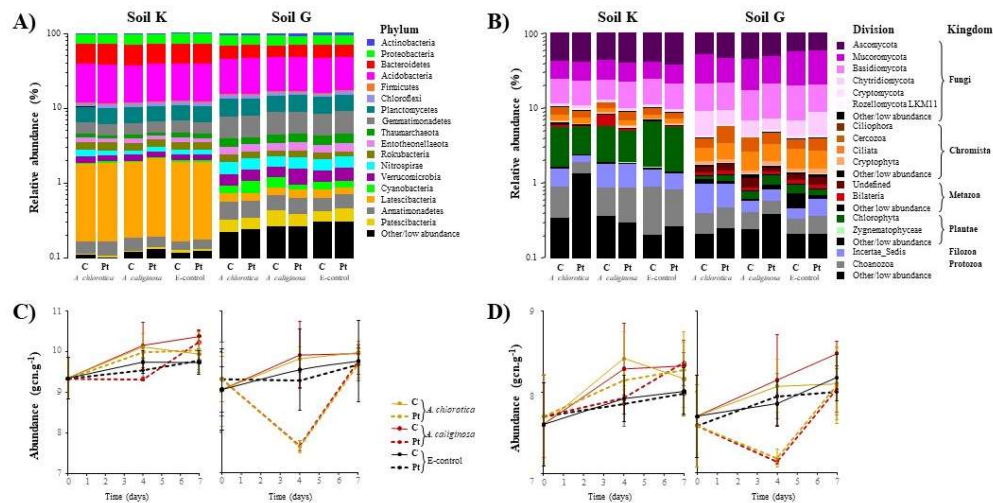
To measure mOTUs differential abundance (DA) across eaten soil and ejected cast communities, we used RStudio (RStudio team, 2020) and the DEBrowser package with the

DESeq2 method (Kucukural et al., 2019) available in the Bioconductor software (Gentleman et al., 2004). Variable indicators of the mOTUs' abundance matrices were informed with log<sub>2</sub> fold change (log<sub>2</sub>FC) values for mOTUs showing significant differential abundance, allowing further selection of upheld (log<sub>2</sub>FC > 0) or depleted (log<sub>2</sub>FC < 0) mOTUs in casts following digestive transit. The third microbiota portion of unchanged abundance following digestive transit was not examined. DA analysis was designed to compare microbiota of all soil samples replicates at the initial sampling time (soil K and G, d0) to each cast's sample replicate of a given experimental condition at the final sampling time (soil K and G, Pt and control soil, d12), yielding four discrete DA analysis for a given experimental condition that allowed ANOVA and PERMANOVA analysis of upheld and depleted assemblages (Table S1; Table S2). Fold change (FC) values of selected DA-mOTUs were used to build up DA-community matrix separately for prokaryotes and for micro-eukaryotes. Dissimilarity analysis based on these DA-communities should reflect the turnover of the soil microbiota due to soil digestion by earthworms.

### 3. Results

#### 3.1. Effect of soil type, earthworm species and parathion exposure on the bulk-soil microbiota

Differences in soil microbiota were observed depending on the soil type (Fig. 1, Table S3). While microbial abundances (Ab) and mOTUs richness ( $H_0$ ) were comparable between soils (except at d4), the soil K was characterized by higher numbers of typical and dominant prokaryotic mOTUs ( $H_1$  and  $H_2$  diversity indices, respectively) than the soil G ( $F=137.1$ ,  $p<0.0001$  and  $F=129.8$ ,  $p<0.0001$  for  $H_1$  and  $H_2$ , respectively). On the contrary, the soil G was characterized by higher numbers of typical and dominant micro-eukaryotic mOTUs than the soil K ( $F=38.2$ ,  $p<0.0001$  and  $F=59.0$ ,  $p<0.0001$  for  $H_1$  and  $H_2$ , respectively). Overall, in the soil K microbial abundances were unaffected by the various experimental conditions (Table S4), while in the soil G microbial abundances increased significantly with time ( $F=56.2$ ,  $p<0.0001$  and  $F=22.8$  with  $p<0.0001$  for prokaryotes and micro-eukaryotes, respectively) and were reduced in the pesticide treated (Pt) soil compared to pesticide control (C) soil ( $F=82.9$  with  $p<0.0001$  and  $F=24.3$  with  $p<0.0001$  for prokaryotes and micro-eukaryotes, respectively). The particular sensitivity to the various experimental conditions in the soil G was mainly related to a significant decrease of abundance after 4 days exposure in the presence of earthworm and pesticide (Fig. 1C, 1D).



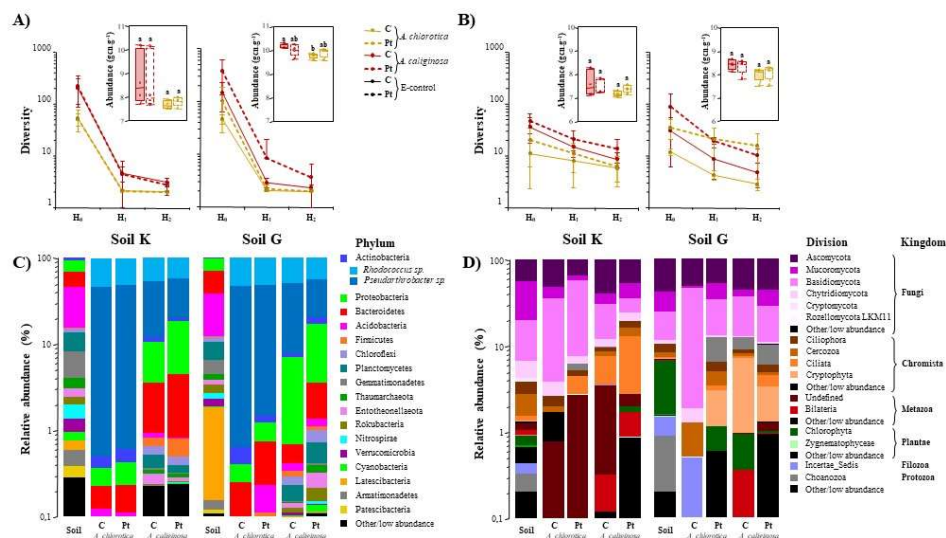
**Figure 1.** Variation of soil prokaryotic and micro-eukaryotic community compositions (A and B, respectively) and corresponding abundances (C and D, respectively) in the soils K and G. For convenience and due to the overall stability of soils microbiota, their composition is displayed following 7 days incubation with *A. chlorotica*, *A. caliginosa* earthworms or without earthworm (EW-free), under control (C) or parathion exposure (Pt). Prokaryotic mOTUs are regrouped according to their phylum affiliation and micro-eukaryotic mOTUs are regrouped according to their kingdom and division affiliation. Both prokaryotic and micro-eukaryotic regrouping are ordered according to their overall relative dominance. The “Other/low abundance” category regroup prokaryotic phyla or micro-eukaryotic divisions having less than 0.1% relative abundance. Variation of microbial abundances is shown across sampling time according to experimental conditions with symbol legend displayed between C and D plots.

The singularity of each soil microbiota was explained by the fractions of prokaryotic and micro-eukaryotic mOTUs shared between soils, representing almost the half of the prokaryotic taxa (6323 out of 13492 ~47%) and the third of the micro-eukaryotic taxa (1202 out of 4369 ~28%), leading to a very high dissimilarity across soils (ANOSIM  $R=1$ ,

$p < 0.0001$ ). Also, soil microbiota showed obvious differences of dominance at prokaryotic phylum and micro-eukaryotic division levels (Fig. 1A, 1B). Measuring the contribution of the various experimental conditions to the turnover of soil microbiota (Table S5) revealed that only the turnover of the micro-eukaryotes was stimulated by the presence of *A. caliginosa* in the soil G (pseudoF=1.52, pMC=0.02; ANOSIM R=0.094,  $p = 0.03$ ) while the turnover of both prokaryote and micro-eukaryote fractions occurred only with time in soil K (PseudoF=1.36, pMC=0.03 and PseudoF=1.42, pMC=0.04 for prokaryotes and micro-eukaryotes, respectively), with not any effect of pesticide.

### 3.2. Effect of soil type, earthworm species and parathion exposure on the earthworms' gut microbiota

Earthworms' gut microbiota varied mostly according to the earthworm species for both the prokaryotic and the micro-eukaryotic fractions (Fig. 2, Table S6). Difference among earthworm species relied on the higher prokaryotic richness in the gut of *A. caliginosa* (~236 mOTUs) compared to that of *A. chlorotica* (~63 mOTUs;  $F = 17.1$ ,  $p = 0.0003$ ) and leading to contrasted diversity profiles between earthworm species (Fig. 2A, Table S6).



**Figure 2.** Variation of abundance, diversity and composition of *A. chlorotica*'s and *A. caliginosa*'s gut microbiota under various experimental conditions: earthworms feeding on either soils K and G, under control or pesticide treated conditions (C and Pt, respectively). The variation of diversity indices and abundances are shown in diversity profiles plots and boxplot inserts for prokaryote (A) and micro-eukaryotes (B) compartments, respectively (see supplementary table S4); abundance values affected with a different letter are significantly different for  $p < 0.05$ . The variation of community compositions in guts compared to bulk soils are shown in barplots for prokaryotic (C) and micro-eukaryotic (D) compartments, respectively (see supplementary table S5); prokaryotic mOTUs are regrouped according to their affiliation at the phylum level (at the exception of Actinobacteria that is dominated by two mOTUs, named at the genus and species levels), and micro-eukaryote mOTU's are regrouped according to their kingdom and division affiliation; both prokaryotic and micro-eukaryotic grouping are ordered according to their overall relative dominance.

For *A. chlorotica*, the gut prokaryotic community was dominated by the single prokaryotic phylum of Actinobacteria and specifically two mOTUs affiliated to species of the genus *Rhodococcus* and *Pseudarthrobacter* (Figure 2C), while mOTUs affiliated to other phyla

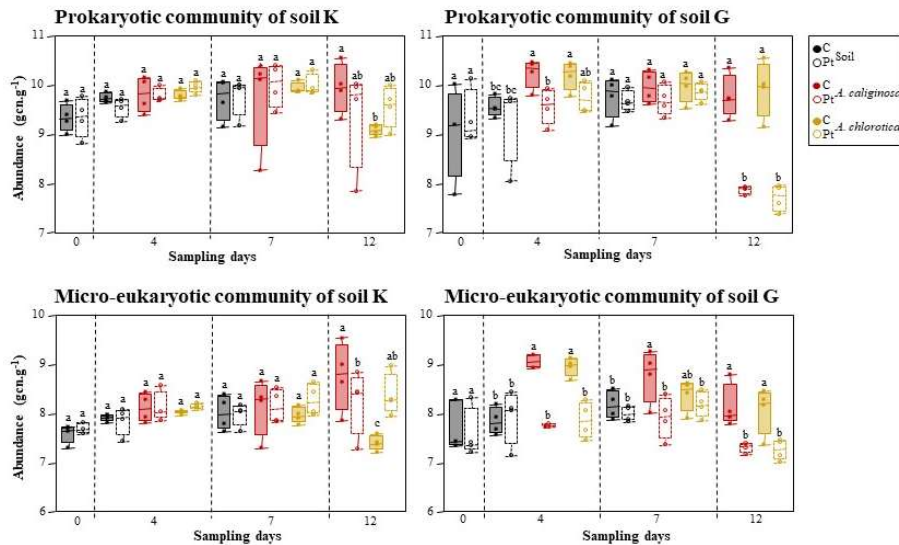
accounted for less than 1% relative abundance. On the other hand, for *A. caliginosa*, beside domination by Actinobacteria, displayed mOTUs affiliated to other phyla that accounted for more than 10% relative abundance, and secondary dominance of mOTUs affiliated to the Proteobacteria and Bacteroidetes (Fig. 2C). The gut micro-eukaryotic fraction also showed higher richness for *A. caliginosa* (~51 mOTUs) than for *A. chlorotica* (~19 mOTUs;  $F=8,0$ ,  $p=0.008$ ), but with lower slope of diversity profiles (Fig. 2B) than for prokaryote (Fig. 2A). The gut micro-eukaryotic fraction of both earthworm species was dominated by the Fungi while mOTUs affiliated to other kingdom accounted for 1 to 10% relative abundance. The gut microbiota showed higher abundances for earthworms placed in soil G compared to soil K for both prokaryotes (~9.95 log gc.n.g<sup>-1</sup> and ~8.15 log gc.n.g<sup>-1</sup> in soil G and K, respectively;  $F=69.3$ ,  $p<0.0001$ ) and micro-eukaryotes (~8.20 log gc.n.g<sup>-1</sup> and ~7.37 log gc.n.g<sup>-1</sup> in soil G and K, respectively;  $F=50.9$ ,  $p<0.0001$ ). Also, independently of the soil and the pesticide treatment, the gut of *A. caliginosa* showed on average higher microbial abundances than that of *A. chlorotica* (~1.15 log gc.n.g<sup>-1</sup> and ~0.91 log gc.n.g<sup>-1</sup> respectively for prokaryotes;  $F=4.3$ ,  $p=0.05$  and ~0.96 log gc.n.g<sup>-1</sup> and ~0.74 log gc.n.g<sup>-1</sup> respectively for micro-eukaryotes;  $F=4.2$ ,  $p=0.05$ ). The micro-eukaryotic diversity was greater when earthworms were exposed to parathion compared to the control, for both earthworm species (one-way repeated measure MANOVA  $F=7.07$ ,  $p=0.02$  for *A. caliginosa* and one-way repeated measure MANOVA  $F=7.57$ ,  $p=0.02$  for *A. chlorotica*).

Concerning the turnover of the gut microbiota (Table S7), we observed that the prokaryotic fraction differed mainly across earthworm species (PseudoF=16.0,  $P_{perm}<0.0001$ ) and was not influenced by the soil type nor by the pesticide treatment. Comparatively, the micro-eukaryotic fraction differed according to the soil origin (PseudoF=2.6,  $P_{perm}=0.001$ ), the earthworm species (PseudoF=2.1,  $P_{perm}=0.002$ ) and the pesticide treatment (PseudoF=1.8,  $P_{perm}<0.0001$ ).

### 3.3. Variability of the earthworms' casts microbiota

The variations of microbial abundances (depending on time, earthworm species and parathion exposure) measured in the earthworms' casts were more pronounced when earthworms were placed in the soil G compared to the soil K (Fig. 3). This is particularly true in the fresh casts collected 5 days after the earthworms were taken out of the soil (12-days endpoint samples; see Table S6). After 4 days of exposure in the soil G and in the absence of Pt, casts samples showed higher prokaryotic and micro-eukaryotic abundances than the control bulk-soil without earthworms. It is noteworthy that in the presence of Pt, both prokaryotic and micro-eukaryotic abundance in casts collected after 4 and 7 days, remained equivalent to that of the

control bulk-soil. However, exposure to parathion led to noticeable reduction of casts' prokaryotic and micro-eukaryotic abundances compared to control casts, and this observation was mostly evident in d12 fresh casts.

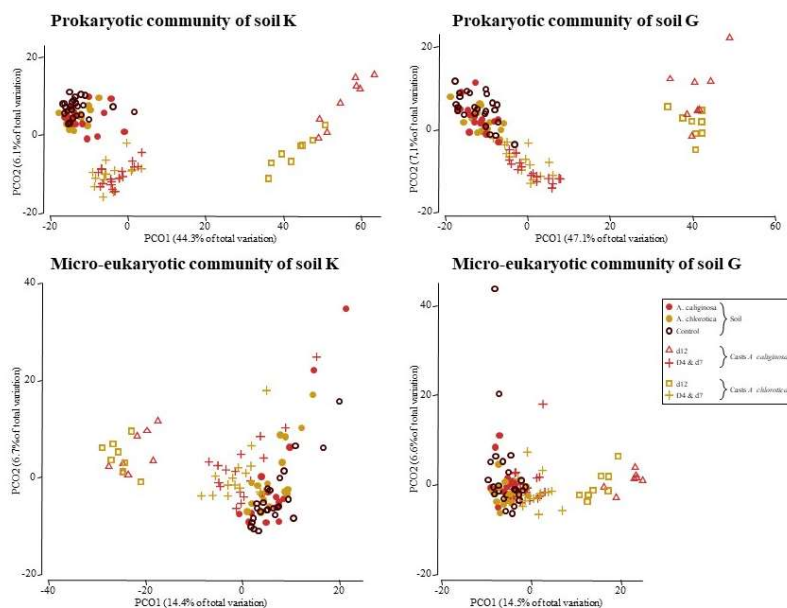


**Figure 3.** Boxplots showing changes of abundance between the bulk-soil (d0) and casts (other dates), for prokaryotic and micro-eukaryotic communities, during feeding of *A. chlorotica* and *A. caliginosa* earthworms on soil K or G. For each date, values affected with a different letter are significantly different with  $P < 0.05$ .

The fresh casts showed greatest reduction of prokaryotic community diversity compared to micro-eukaryotic communities (Fig. S1; Table S6). Also, *A. caliginosa*'s casts showed globally lower prokaryotic diversity than those of *A. chlorotica*, which varied according to the type of soil. An overall pesticide effect was recorded in relation to the decrease of micro-eukaryotic diversity in casts that must be considered together with the increase of micro-eukaryotic diversity in gut (Table S6; Fig. 2).

The ingested soil had obviously undergone changes during transit in the earthworm intestine up to defecation, which was evidenced by dissimilar soil and casts microbiota (Fig. 4; Fig. S2). Fresh casts, collected on filter papers after 12 days experimental procedure, were most dissimilar to the soil microbiota, while ageing casts (d4- and d7- samples) that remained several days on the soil, showed less dissimilarity to the corresponding bulk-soil microbiota. Both prokaryotic and micro-eukaryotic communities of fresh casts showed important dissimilarities between soil types (PseudoF=70.6,  $P_{perm} < 0.0001$  for prokaryotes and PseudoF=52.7,  $P_{perm} < 0.0001$  for micro-eukaryotes) and between earthworm species (PseudoF=4.7,  $P_{perm} < 0.0001$  for prokaryotes and PseudoF=2.03,  $P_{perm} = 0.0007$  for micro-eukaryotes; Table S5), which underlined the fact that i) community composition of the fresh casts and that of the eaten soil show strong relationships from a same soil and a same earthworm species and ii) each earthworm species displays different digestive transit. Furthermore, in the soil K, modification of casts' microbiota due to parathion exposure

revealed only limited effects on the micro-eukaryotic fraction (PseudoF=1.4, Pperm=0.03; Table S7).



**Figure 4.** Diversity changes between soil and casts prokaryotic and micro-eukaryotic communities, during feeding of *A. chlorotica* and *A. caliginosa* earthworms on soil K or G. Dissimilarity across samples is shown through Principal Coordinate Ordination plots. Focus is made to distinguish freshly dejected casts samples collected after 12-days (12d) from old casts samples collected after 4- or 7- days (4d and 7d, respectively) experimental incubation.

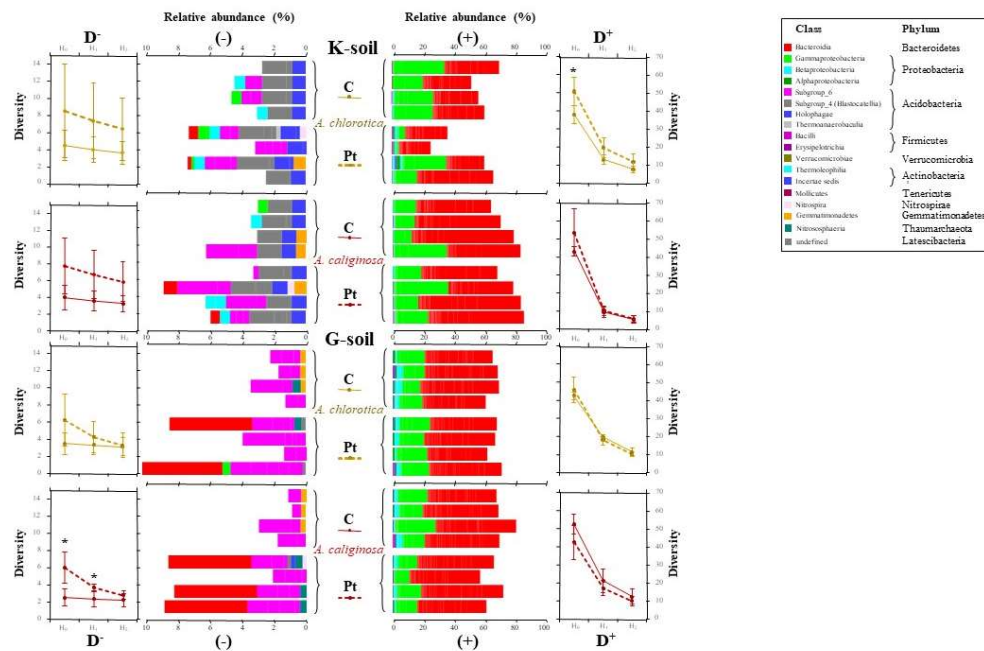
### 3.4 Upheld- and depleted- soil microbial mOTUs in fresh casts after gut transit

Differential abundance (DA) analysis across control soil and fresh casts allowed identifying microbial mOTUs showing higher or lower abundances in the fresh casts than in the corresponding bulk-soil (upheld- and depleted- mOTUs, respectively; TableS1 and S2, Tables S8 andS9). More variability was obtained to assess dissimilarity among soil and casts samples based on the sub-selection of upheld- and depleted- mOTUs (Fig. S2) than when considering all mOTUs obtained from the amplicon sequencing (Fig. 4). DA analysis allowed dividing the soil microbiota into three distinct portions: (i) depleted mOTUs that are present in soil but absent or having reduced abundances in casts, suggesting their consumption as a nutritive resource by earthworm, (ii) upheld mOTUs that are present in cast but absent or having reduced abundances in soil, suggesting they are subject to stimulation/selection process and (iii) mOTUs that had not shown any change of abundance following gut transit (not considered here).

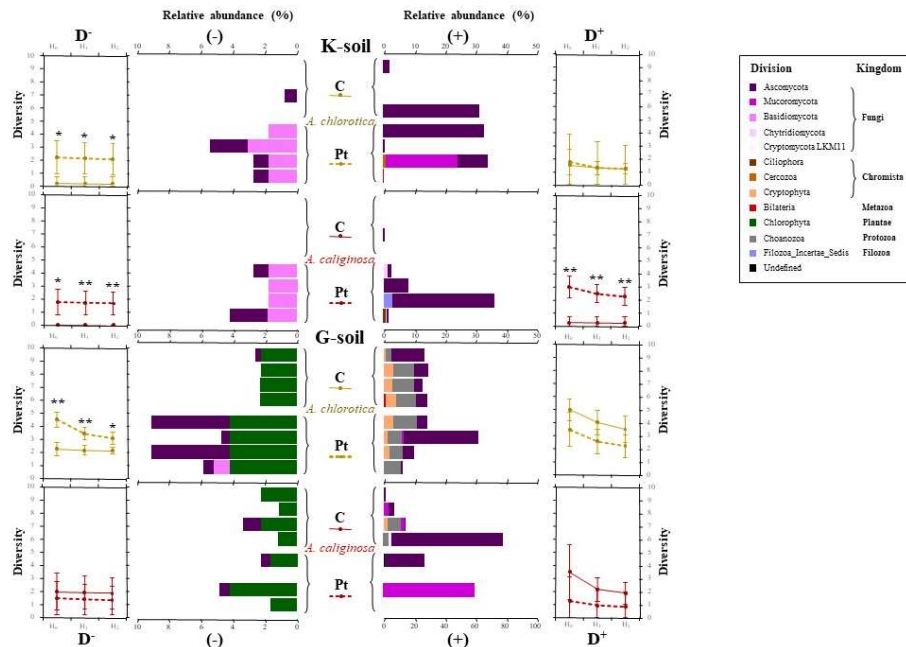
Upheld prokaryotic mOTUs were on average more numerous (ranging from 32 to 72 mOTUs per fresh cast sample) than depleted mOTUs (ranging from 2 to 13 mOTUs per fresh cast sample), leading to diversity profiles with higher values in upheld assemblages than in depleted assemblages (Fig. 5). Examining the pesticide-free condition, the composition of upheld- and depleted- prokaryotic assemblages showed interesting differences at phylum and class taxonomic levels. In both soils, Bacteroidia and  $\gamma$ -proteobacteria dominated in upheld



prokaryotic mOTUs, whereas depleted mOTUs were affiliated to diverse subgroup of the phylum Acidobacteria, with dominance of subgroup 6 in the soil G.



**Figure 5.** Diversity of differentially abundant prokaryotic mOTUs in fresh casts, under the various experimental conditions: *A. chlorotica* and *A. caliginosa* earthworms feeding on either soil K and G, under control or pesticide treated conditions (C and Pt, respectively). Prokaryotic mOTUs are regrouped according to their affiliation at the phylum and class levels. (+) and (-) symbols show relative abundance of prokaryotic mOTUs that are upheld in casts and depleted in soil, respectively; D- and D+ displayed aside, show corresponding diversity profiles under control or pesticide treated conditions. Significant differences across conditions are indicated with the symbol \* for  $P < 0.05$ .



**Figure 6.** Diversity of differentially abundant micro-eukaryotic mOTUs in fresh casts, under the various experimental conditions: *A. chlorotica* and *A. caliginosa* earthworms feeding on either soil K and G, under control or pesticide treated conditions (C and Pt, respectively). Prokaryotic mOTUs are regrouped according to their affiliation at the phylum and class levels. (+) and (-) symbols show relative abundance of prokaryotic mOTUs that are upheld in casts and depleted from soil, respectively; D- and D+ displayed aside, show corresponding diversity profiles under control or pesticide treated conditions. Significant differences across conditions are indicated with symbols \* for  $P < 0.05$  and \*\* for  $P < 0.01$ .

In the absence of pesticide exposure, the variation of upheld and depleted micro-eukaryotic mOTUs showed different patterns than those of prokaryotes (Fig. 6): (i) both upheld and

depleted micro-eukaryotic mOTUs were very rare (from 0 to 6 mOTUs per fresh cast sample), leading to diversity profiles with low values in both upheld- and depleted-assemblages and (ii) the composition of these assemblages was influenced by the soil origin and slightly by the earthworm species (Fig. 6). In the soil K, both upheld- and depleted-assemblages (strictly affiliated to the phylum Ascomycota) were poorly recorded for *A. chlorotica*, while none were found for *A. caliginosa*. In the soil G, the composition of upheld and depleted micro-eukaryotic mOTUs showed minor differences across earthworm species. However, upheld mOTUs were affiliated to several kingdoms including Fungi (Ascomycota), Chromista (Cryptophyta) and Protozoa (Choanozoa), while depleted mOTUs were almost strictly affiliated to Plantae (Chlorophyta).

The exposure to parathion increased the diversity of both prokaryotic and micro-eukaryotic mOTUs that were depleted from soil during transit along the earthworms' gut (Fig. 5, 6; Table S1, S2), and this effect was more pronounced in the soil K than in the soil G. Pesticide exposure had marginal effects on microbial mOTUs upheld in casts that differed according to the soil origin: prokaryotic richness and micro-eukaryotic diversity increased in the soil K, while micro-eukaryotic diversity was reduced in the soil G.

#### **4. Discussion**

In our study, the comparative analysis of soil, gut and cast microbiota enabled us to assess the influence of environmental changes during the earthworm gut transit on the dynamics of soil microbial communities. We have found higher prokaryotic content in the silt-loamy soil (soil K) and higher micro-eukaryotic diversity in the silt-clay soil (soil G). This is consistent with previous works showing that the soil texture is a key driver of the diversity and composition of soil microbial communities (Wang H. et al., 2021) and affects the competitiveness of soil microbes according to their life history traits (Mirleau et al., 2001; Latour et al., 2009). Moreover, Seaton et al. (2020) pointed out that the fungal community is subject to an indirect effect of texture mediated by the prokaryotic community.

##### *4.1. Prokaryotic simplicity and micro-eukaryotic complexity of the gut microbiota*

We observed that the microbial composition of the earthworms' gut depends on earthworms' species as previously evidenced (Sapkota et al., 2020) but could be slightly influenced by the soil type. We can hypothesize that the acclimatization period of our experiment was too short to initiate a change of the gut microbial composition according to the composition of the soil in which they don't usually live (Wang N. et al., 2021). The difference observed between earthworm species is probably linked to their habitual soil habitat. In any case, we evidenced

the simple composition of the prokaryotic fraction of the gut microbiota compared to the micro-eukaryotic fraction.

Two prokaryotic mOTUs affiliated to species of the genus *Rhodococcus* and *Pseudarthrobacter*, showed selective enrichment with relative abundances rising from less than 1 % in soils to more than 40 % in guts. Members of these prokaryotic genus have previously been identified as dominant prokaryote of endogeic earthworm's gut wall (Thakuria et al., 2010, Sapkota et al., 2020) or showing highest increase in abundance during gut transit (Aira et al., 2022b). The relative abundance of these mOTUs reached above 99% in *A. chlorotica*'s gut and about 90% in *A. caliginosa*'s gut, supporting the idea of an established symbiotic relationship as previously evidenced for the endogeic earthworm *Onychochaeta borincana* (Valle-Molinares et al., 2007). However, *A. caliginosa*'s gut showed a more diverse prokaryotic community affiliated to several phyla that correspond to the eaten soil compared to the much simpler prokaryotic community of *A. chlorotica*'s gut. This observation could be related to differences in gut fluid activities (Khomyakov et al., 2007) that could vary according to earthworm species, driving more permissive or more drastic conditions that favor or inhibit prokaryotes.

The micro-eukaryotic fraction of the gut microbiota showed more equilibrium between mOTUs' relative abundances. In guts and soils, the most abundant mOTUs (~90 %) were affiliated to several Fungi divisions, while rare mOTUs (~10 %) were affiliated with all other kingdom in relation to the ingested soil. Fungal dominance in soil or in the earthworm's gut microbiota could be explained by (i) fragmentation of fungal hyphae that multiplies the copy numbers of ITS sequences of a given fungus and (ii) a possible PCR primer specificity bias that favors the amplification of the fungal ITS, both resulting in the lower detection of other unicellular/unfragmented eukaryotes. However, several clades other than Fungi showed particularly high relative abundance in the gut compared to the corresponding soil, independently of the earthworms' species. Besides Fungi, most dominant mOTUs (>1%) were affiliated to ameboids of the divisions Ciliata (genus *Telotrochidium*) and Cercozoa (genus *Gymnophrys*) with both soils; mOTUs affiliated to the division Ciliophora (order Geotrichida) were typically counted with the soil K, while mOTUs affiliated to the division Choanozoa (of the Ichtyophonidae and Codosigidae families) and Cryptophyta (Genus *Plagioselmis*) were typically counted with the soil G. Much other scarce divisions recorded in soils microbiota decreased importantly in the earthworms' gut, suggesting they were counted as cellular remains in soils, further degraded and/or consumed. Overall, peculiar resistance forms of these organisms, such as spores, cysts, and eggs that would maintain tightly attach to

the intestine wall, possibly explain the maintenance of a diversified eukaryotic microbiota in the earthworms' gut, despite cleaning steps.

#### 4.2. *The digestive transit: an environmental filter for soil microbes*

For both earthworm species incubated in the soil G, gut microbial abundance was the highest, while the lowest in their fresh casts. This result points out the earthworms' preferences to retain and digest some microorganisms from soil G. Previous studies have shown that the effect of earthworms on soil microorganisms depends on the kind of ingested food (Flegel and Schrader, 2000; Tiunov and Scheu, 2000) and that microorganisms constitute an important and selective part of the earthworms' diet (Moody et al., 1995; Brown et al., 2004). Here, among obvious differences between the two soils, algae (Chlorophyta) are more dominant in the soil G than in the soil K and one mOTU affiliated to *Actinastrum hantzschii* was specifically depleted from the soil G following digestion (Table S7), while very few micro-eukaryotic mOTUs were retained after gut transit of the soil K. This result agrees with the recent demonstration that feeding on photoautotrophic soil surface microorganisms is an important carbon input for earthworms (Schmidt et al., 2016). Diet preference was less obvious for prokaryote. It appeared that Acidobacteria subgroup 6 was the most dominant in the soil G that was retained during digestion while those retained from the soil K belonged to several subgroup of Acidobacteria and other prokaryotic phyla such as Actinobacteria. The targeted subgroup 6 of Acidobacteria which was depleted from soil G may be related to its vulnerability to the shift from aerobic to anaerobic conditions or to the degradation of cell membrane mediated by fluid and enzymes secreted in the intestine (Byzov et al., 2007; Brown et al., 2000). Overall, the fraction of the soil microbiota depleted during intestinal transit represented less than 5% for both earthworm species, even though each species thrived preferentially in its original soil. This result comforts the view that these endogeic species have similar opportunistic diets independently of the soil habitat they thrive in.

Fresh casts possessed a very specific community composition, compared to older casts that showed a close resemblance to the ingested soil. This can be explained by two mechanisms. Firstly, ageing casts (4d- and 7d- samples), by remaining several days on the soil, were subject to microbial recolonization. This process was rapid and likely occurred along with decreasing content of labile carbon and nitrogen pool during cast ageing (Aira et al., 2019). Secondly, numerous microorganisms go through the earthworm digestive tractus without being affected (Drake and Horn, 2007). In any case, the cast microbiota showed higher abundances than the initial bulk-soil, highlighting the stimulation of microbial growth

following intestinal transit even after recolonization by soil microbes. Prokaryotic growth stimulation has been attributed to anaerobic and fermentative microbial activities involved in the catabolism of large molecules and in the stimulation of carbon, nitrogen and phosphorus cycles (Lubbers et al., 2017; Budroni et al., 2020; Wang N. et al., 2021). This stimulation could be linked to higher soil enzyme activities measured in casts compared to the initial bulk-soil (Jouni et al., 2023).

While the stimulation of prokaryotic mOTUs was mainly dependent of the ingested soil, that of micro-eukaryotic mOTUs also differed according to the earthworm species, suggesting that the stimulation processes through the gut transit differ between prokaryote and micro-eukaryotes. Stimulation of prokaryote has been shown to be favored by either the availability of labile organic matter in casts (for  $\gamma$ -proteobacteria; Braga et al., 2016), the anaerobic conditions of the earthworm's gut (for facultative anaerobes such as denitrifiers; Ihssen et al., 2003) or the specific abilities to utilize cellulose degradation products such as cellobiose (Karsten and Drake, 1995). Our results showed these bacteriological characteristics are related to the composition of the bulk-soil prokaryotic communities rather than earthworm species as previously suggested (Jacquiod et al., 2020). Contrastingly, micro-eukaryotic stimulation differed between earthworm species raises questions on possible selective stimulation attributed to the intestinal fluid (Byzov et al., 2007).

#### *4.3. The effects of ethyl parathion on the earthworms mediated filtering of soil microbes*

Interestingly, the effects of the pesticide treatment seemed marginal. Among visible effects, ethyl parathion produced (i) a temporary decrease of soil microbial abundances, (ii) an increase of the gut micro-eukaryotic diversity and (iii) a decrease of casts microbial abundances and diversity that expressed more importantly with the G-soil. Other studies have shown contradictory effects of pesticides on gut microbiota: imidacloprid (neonicotinoid), benomyl (fungicide) and metribuzin (herbicide) were shown to reduce the total prokaryotic diversity in the earthworm's gut (Astaykina et al., 2022). However, we can assume that the soil texture could affect the response of microbial communities as a weak restoration of enzyme activities was observed after parathion exposure in casts from soil G (Jouni et al., 2023). The pesticide effects were more marked when considering fractions of the soil microbiota that were upheld or depleted following digestive transit. With both soils and both earthworm species, the increasing diversity of depleted mOTUs related to ethyl parathion, could rely on the stimulation of microbial lysis during digestion. For instance, the increased diversity of digested microbiota involved prokaryotic taxa affiliated to the Bacteroidetes

(class Bacteroidia) and microeukaryotic taxa affiliated to the Fungi (Division Ascomycota and Basidiomycota) that were not recorded as part of the earthworms' diet in the absence of pesticide (see section 4.2). Modification of the fraction of the soil microbiota upheld in casts was more erratic and mainly evidenced for microeukaryotes upheld in casts of *A. caliginosa*.

## 5. Conclusion

This study emphasizes that the gut prokaryotic community composition mainly differs according to the earthworm species, with *A. chlorotica* showing a large dominance of rare mOTU's, while *A. caliginosa* exhibits more numerous mOTUs from the eaten soil.

Subsequently to the intestinal transit, microbial communities from fresh casts differ from those of initial bulk soils. The stimulation of particular prokaryotic mOTUs is regular across conditions, while that of micro-eukaryotic mOTUs depends on the earthworm species, which points out a species-dependent role of the intestinal mucus in the relationship with soil micro-eukaryotic communities.

Ethyl-parathion appears to mostly affect the soil and casts microbial communities compared to those of the gut. The decrease of microbial abundance and diversity in casts, particularly observed in the silt-clay soil G, must be put in relation with our previous work (Jouni et al., 2023) where soil enzyme activities were more affected by ethyl-parathion in that soil.

Lastly, ethyl-parathion appears to modify the digestive capacities of earthworms by enhancing the diversity of prokaryotic and micro-eukaryotic mOTUs depleted during the gut transit compared to unexposed earthworms. Organophosphorus pesticides are known to inhibit acetylcholinesterase resulting in rapid accumulation of the neurotransmitter acetylcholine. As an excess of acetylcholine could provoke an over excitability of the gastrointestinal tract (Krajniak and Klohr, 1999), this result calls for further investigations on the effects of pesticides on the intestinal contractions of earthworms, their role on microbial digestion and/or on the production of intestinal mucus.

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