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Investigating the effects of nitrogen deposition and substrates on the microbiome and mycobiome of the millipede *Cherokia georgiana georgiana* (Diplopoda: Polydesmida)

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ABSTRACT

Millipedes are ubiquitous soil invertebrates that play major roles in physical and chemical processes in soil. Despite their importance to soil ecology and their presumed interactions with soil microorganisms, little is known about how millipedes influence and are influenced by soil microbes. Furthermore, it is not fully understood how these millipede-microbe interactions are influenced by available soil nitrogen, which is predicted to increase over the foreseeable future with increased anthropogenic production. Here, using the millipede *Cherokia georgiana georgiana* as a model and using a manipulative mesocosm and metabarcoding approach, we examine (1) the impacts of millipedes on soil microbial communities (fungi and bacteria) with varying nitrogen addition levels, and (2) the temporal impacts of nitrogen on millipede fecal communities (fungi and bacteria). This research demonstrates that millipede presence strongly alters soil communities and that alterations in nitrogen levels do not impact millipede gut communities. This work also provides the first evidence suggesting that millipede gut communities are predominantly derived from soils rather than leaf litter, though both contribute to gut composition. This work advances our current poor understanding of millipede-soil interactions and provides a framework for further investigations to disentangle the interactive effects of substrate, nitrogen, and time to better understand ecological impacts of these interactions.

1. Introduction

Millipedes (Class Diplopoda) are ubiquitous, widely distributed, and diverse arthropods, with ~15,000 currently accepted species belonging to near 3000 genera (Sierwald and Bond, 2007; Sierwald and Spelda, 2020). They are widely accepted to be important in soil processes (e.g., decomposition and calcium cycling, Coleman and Wall, 2015) and maintaining soil fertility (Culliney, 2013). They are the dominant soil macrofauna in some forested communities, especially where a developed humus layer consists of hardwood derived mull and mor types (Culliney, 2013; Raw, 1967). Millipedes are most well known for their role in decomposition processes, mainly through comminution (Hopkin and Read, 1992). However, quantitative data on their impacts are lacking, leaving their ecological contributions poorly understood. Where these data do exist, most studies have focused on leaf litter consumption for individual species (Coq et al., 2018; Joly et al., 2020; Steinwandter and Seeber, 2020). Consumption can be as high as 39% of the standing stock of leaf litter (Dangerfield and Milner, 1996) but this is highly variable depending on species, the decomposer community, and the density of the millipedes. Millipedes can be thought of as broad generalist decomposers in most systems (Semenyuk et al., 2011; Steinwandter and Seeber, 2020), which may include soil in addition to leaves (Dangerfield, 1993). However, it is yet to be resolved if millipedes actively target consumption of soils or if this consumption is incidental with leaf litter consumption, but significant soil particulate matter percentages have been found in millipede fecal pellets (Mwabvu, 2018).

Further, while millipedes are undoubtably important for soil development and turnover, mainly due to their role in decomposition processes (Seeber et al., 2006), direct impacts of millipedes on soil biogeochemical processes remain poorly resolved. Soil invertebrates as a

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whole (including millipedes) are known to play a large role in structuring rhizospheric microbial communities and can increase fungal and bacterial biomass, and well as eliciting an increase in phenol oxidase and glucosidase activities (Bray et al., 2019). Additionally, under controlled laboratory experiments, the density of the millipede *Glyphiulus granulatus* (Polydesmida; Paradoxosomatidae) has been demonstrated to be directly linked to reduced soil aggregation, increased nitrogen, decreased C:N, increased activity of β-glycosidase, and a decrease in acid phosphatase activity (da Silva et al., 2017). Millipedes have also been shown to release significant amounts of the greenhouse gasses CO_2, CH_4, and N_2O (Šustr et al., 2020a,b) possibly because of tight millipede-gut microbial linkages as seen in other terrestrial arthropods (Hackstein et al., 2006).

However - and despite millipedes' ubiquity - little research exists using modern molecular tools to assess millipede-microbial interactions. What work has been conducted on describing millipede-associated microbes has been primarily culture and microscopy based (*reviewed in* Byzov, 2006). An additional confounding factor to understanding millipede microbiomes is that millipede diets are not well characterized and only known in general terms. Millipedes consume microbes with their food sources, and likely incidentally derive energy from these, yet other microbes can presumably evade digestion, become gut residents, and thrive in various gut environments (Byzov, 2006; Hopkin and Read, 1992). Assessment of fecal microbial communities may inform questions about both diet and roles in ecosystem processes.

In addition to unresolved questions on millipede gut microbial communities, the impacts and implications of millipede activity on soil communities is understudied, particularly under the framework of differential soil chemistry. With an increasingly industrialized world, we are facing changes in anthropogenic nitrogen deposition (Oulehle et al., 2016) which may increase or decrease in the Eastern US based on current regulatory policies (Gilliam et al., 2019; NADP, 2016; Reay et al., 2008). This, along with changing atmospheric CO_2 levels, will alter forest soils and nutrient loads, and ultimately impact primary productivity (Stevens et al., 2015). With warming, millipede populations are predicted to increase globally (*but see* Snyder and Callaham, 2019). Coupled with different leaf and soil quality due to elevated atmospheric CO_2 (David, 2009), millipedes will likely play increasingly important roles in soil processes. Increased anthropogenic nitrogen deposition is also strongly correlated with declines in plant richness, with a generalized reduction in native forb richness with a contaminant increase in nonnative plant cover driving this pattern (Valliere et al., 2020). This reduction in richness and plant species turnover will likely facilitate changes in leaf-litter diversity and chemistry, which will have unknown impacts on litter decomposers such as millipedes. In general, how increased nitrogen inputs affect soil fauna and how the interactions between nitrogen inputs and soil fauna affect soil biology is understudied. Investigations that examine and integrate the roles of millipedes and N-enrichment on soil ecology and how N-enrichment in soils impact millipede gut communities will advance our current poor understanding of how millipedes help shape soils and how soils and/or leaf litter in turn shape millipede microbiomes.

To investigate these issues, *Cherokia georgiana georgiana* (Bollman, 1889) was chosen as a model to understand how millipedes might affect soil microbial communities and how soil and/or leaf litter might affect millipede associated microbes. *C. g. georgiana* belongs the most diverse millipede order (Polydesmida); its family, Xystodesmidae, is one of the most diverse polydesmid families and has a holarctic distribution (Marek et al., 2014; Shelley and Smith, 2018). *Cherokia* spp. are abundant and widespread across the Southern Appalachian mountains of the United States (Hoffman, 1960), which is also the center of xystodesmid diversity (Means and Marek, 2017). Due to the large biomass and abundance of *C. g. georgiana* in southern Appalachian forest ecosystems, they likely have significant effects on soil processes in this region.

We used a mesocosm framework across a time series with manipulated soil nitrogen levels to interrogate three important but hitherto unresolved questions about millipede, nitrogen, and soil interactions. Specifically, we ask the following questions: 1) Are soil microbial communities sensitive to millipede presence, nitrogen additions, time, and associated interactions and are these changes similar for bacterial and fungal communities? Given that millipedes are likely an important contributor to soil processes, we predict that soil microbial communities will be impacted by millipede occurrence, but existing literature does not to allow us to make a prediction on what this impact might look like. 2) Do millipede fecal communities change across nitrogen levels and/or time for bacteria and fungi? This question is unstudied, so predictions are difficult. However, if is reasonable to assume that gut communities might be sensitive to outside chemical conditions (nitrogen), unless nitrogen conditions are seen to play a minimal role in food consumption. 3) Can we provide evidence for where fecal microbes originate from, i.e., are they more derived from soil or leaf litter, and is this the same for fungi and bacteria?

2. Materials and methods

2.1. Overview

To investigate the joint effects of millipede presence and nitrogen levels on soil microbial communities and what drives variations in millipede gut microbiome communities, we conducted a time series mesocosm experiment. To address the following main objectives, we analyzed relevant experimental units separately: Objective *1 - Millipede and Nitrogen Amendment Impacts on Soil Microbial Communities, Objective 2 - Millipede Microbiome Modifications with Nitrogen Levels*.

2.2. Experimental design and mesocosm setup

The experiment was conducted using a full factorial design with three levels of nitrate additions (ambient, +10 kg/ha, +20 kg/ha) and two levels of millipede occurrence (a single individual present or absent). Each treatment combination was replicated 10 times for a total of 60 mesocosms. Mesocosms were incubated (Thermo Fisher Precision PR505755L) for 30 days at 20 °C, with a 16 h on/8 h off light cycle. Mesocosms consisted of polypropylene specimen cups (7 cm top diameter, 6.5 cm depth, Fisher Scientific 14-955-114B) with lids perforated with pin holes. Each mesocosm received 65.0 g air-dried soil and 1.0 g air-dried coarse chopped *Acer rubrum* leaf litter. Leaves were collected from under a single tree in a residential backyard in rural Baldwin Co., Georgia, USA.

2.3. Millipede sourcing

Millipedes were collected from Georgia College's Lake Laurel Biological Station in Baldwin Co., Georgia, USA by hand searching in leaf litter. All millipedes were identified as *Cherokia georgiana georgiana* based on color pattern, size, and other somatic characteristics. Millipedes were weighed and sexed before the start of the experiment, and randomly sorted into nitrate treatments. Millipede survival was assessed every 2–3 days and mesocosms were misted with water on a weekly basis to keep the millipedes in a moist environment. If millipedes died before the end of treatment, their bodies were removed from mesocosms and frozen for later analysis. Survival did not vary across our experiment and only 2 of 30 individuals died during the experiment.

2.4. Soil composition and analyses

Soils were collected from Georgia College's Lake Laurel Biological Station in Baldwin Co., Georgia, USA. Soils were sieved through a 2 mm mesh to remove rocks, macroaggregates, sticks, mesofauna, and macrofauna. Bulk, sieved soil was homogenized and separated into three portions. Two portions were amended with sodium nitrate (Acros Organics) to simulate levels of nitrogen deposition equivalent to +10 kg/

ha and +20 kg/ha, high levels for the region and continent, respectively. Soil was spread out, sodium nitrate was spread throughout the soil, and the soil was thoroughly mixed. Soils were rested for 48 h and thoroughly mixed again before being placed into mesocosms. Soil for the 'ambient' treatment was also subjected to this same procedure without nitrate addition. Nitrate concentration in the soils were measured at the experiment start and Days 5, 20, and 30 by placing 1 g soil in 5 mL deionized water and measuring with a nitrate ion-selective electrode (Cole-Parmer EW-27504-22).

2.5. Sample collection

For sequence generation (section 2.7), soils, leaf litter and fecal material were collected at days 10 and 30 from each mesocosm (n = 60, 180 total samples). Sufficient amount of each material was collected to fill a sterilized 2 mL microcentrifuge tube. Collection tools were cleaned between each sample. Soils were collected using a 10 mL infant medicine syringe with the tip removed. This functioned as a miniscule soil corer (inside diameter 9 mm, maximum possible core depth 48 mm). A portion of the soil surface was exposed, and the device was pushed into the soil. The syringe's plunger was used to push the soil into the microcentrifuge tube. Leaf litter samples were haphazardly collected by forceps. Xystodesmid millipedes often press their fecal material onto surfaces, leaving a characteristic print of their anal valves. Fecal material samples were scraped primarily from the mesocosm sides with forceps or a small spatula. All samples were frozen and kept at −80 °C and shipped to the University of Memphis for DNA extraction and sequencing library preparation.

2.6. DNA extraction

Genomic DNA was extracted from soil, fecal, and litter samples using DNeasy PowerSoil Kit (QIAGEN, Germantown, MD, USA) following the manufacturer's protocol with one modification for litter samples; prior to extraction, leaf litter was ground using a handheld spice grinder to mechanically shred litter prior to extraction, which was sterilized between samples. Extracted DNA was quantified on a NanoPhotometer N60 (Implen, München, Germany) and normalized to a concentration 5 ng μL^{-1} prior to amplification.

2.7. Library generation

Amplicon libraries were generated by amplifying the bacterial 16S (V4) region and the fungal Internal Transcribed Spacer Region 2 (ITS2) of the rRNA gene repeat using a two-step amplification process (*following* Brown et al., 2018). Target gene regions (ITS2 or V4) were amplified using the primer pairs nexF-N[3–6]-{fITS 7 or 515f} and nexR-N[3–6]-{ITS4 or 806r} where fITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990) are fungal specific primers and 515f and 806r (Caporaso et al., 2011) are bacterial specific primers, N[3–6] represents four identical primers but contain ambiguous nucleotides (3–6) mixed to equal molarity, and nexF and nexR are Nextera forward and reverse sequencing primers.

Primary PCR (1°) was conducted in 50 μL reactions using 10 μL DNA template (50 ng), 10 μL 5× Phusion High-fidelity Buffer, 200 μM each dNTP, 0.5 μM of each forward and reverse primer, 0.5 μL Phusion HotStart II DNA Polymerase (0.02 U μL^{-1} final concentration), and 15 μL molecular grade water. The PCR parameters were 98 °C for 30 s, 25 cycles of 98 °C for 20 s, annealing temperature of 52.5 °C for 30 s for bacteria and 51 °C for 30 s for fungi, and 72 °C for 40 s, followed by a final extension at 72 °C for 10 min, all ramp rates were 1 °C s^{-1}. This resulted in a final 1° PCR construct of nexF-N[3–6]-primer-{ITS2 or V4}-primer-N[3–6]-nexR. All 1° PCR products were visually confirmed using gel electrophoresis (1.5% agarose *w:v* in TBE). Secondary PCR (2°) was conducted in 50 μL reactions using the forward primer construct P5-i5-overlap and the reverse primer construct P7-i7-overlap where P5 and P7 are the Illumina Adaptor sequences, i5 and i7 are 8 bp unique Molecular Identifiers (MIDs) and the overlap is the partial nexF and nexR sequence that acts as the annealing site for the 2° PCRs. Forward and reverse 2° primers were mixed in a combinatorial fashion to generate unique dual barcoded primers in a working concentration of 10 μM (5 μM for each primer). The 2° PCR reactions included 10 μL 1° PCR product, 10 μL 5× Phusion High-fidelity Buffer, 200 μM each dNTP, 0.5 μM of each forward and reverse primer, 0.5 μL Phusion HotStart II DNA Polymerase (0.02 U μL^{-1} final concentration), and 20 μL molecular grade water with the PCR parameters of 98 °C for 30s, 8 cycles of 98 °C for 20s, 50 °C 30s and 72 °C for 40 s, followed by a final extension at 72 °C for 10 min. This produced the final amplicon constructs of P5-i5-nexF-N[3–6]-primer-{ITS2 or V4}-primer-N[3–6]-nexR-i7-P7 using a total of 32 cycles. Final PCR constructs were cleaned using Axygene AxyPreg Mag PCR clean-up beads (Axygen Biosciences, Union City, CA, USA) using a 1:1 bead solution to reaction volume (Brown and Jumpponen, 2014). Negative controls were included and were free of observable amplification via gel electrophoresis. Cleaned PCR products were quantified using Qubit 3.0 fluorometric assays (dsDNA HS Assay Kit; ThermoFisher Scientific). Fungal and Bacterial PCR products were separately pooled into libraries at equal concentrations. The amplicon libraries were sequenced on one Illumina MiSeq (v.3, 300 PE) reaction at the Kansas State University Integrated Genomics Facility (Manhattan, KS, USA). Demultiplexing of the raw sequence data using the unique i5 and i7 sequence combinations provided individual paired fastq files for each sample (see Table S1 for primer and MID sequences).

2.8. Bioinformatics

Sequence data were processed using the program mothur (v.1.41.3; Schloss et al., 2009). Individual fastq files for each experiment, and for Fungi and Bacteria, were analyzed separately. Forward and reverse sequences were contiged and screened to cull sequences with ambiguous bases or greater than 12 homopolymers. Contigs were merged into a single fasta file for fungi and bacteria for each experiment and primers were trimmed. Bacterial sequences were aligned against the SILVA reference alignment (release 132; www.arb-silva.de) and filtered to exclude non 16S V4 regions (fungal ITS sequences cannot be reliably aligned). Sequences were preclustered (Huse et al., 2010), screened for chimeras using the mothur implementation of VSEARCH (Rognes et al., 2016), and putative chimeras were culled. Sequences were screened for off-target amplification (non-fungal or non-bacterial in origin) by classifying all sequences using a mothur implemented Naïve Bayesian Classifier (Wang et al., 2007) against the RDP training set (bacteria; v.10) or a UNITE non-redundant species hypothesis dataset (fungi; v6; Kõljalg et al., 2013). Non-target lineages were culled and distance matrices for bacteria (not punishing terminal gaps) were generated. Sequences were clustered into operational taxonomic units (OTUs) at a 3% dissimilarity threshold using OptiClust (Westcott and Schloss, 2017) for bacteria and with VSEARCH (abundance-based greedy clustering) for the fungal datasets. OTUs with fewer than 10 sequences globally were considered potentially spurious and culled (Brown et al., 2015; Oliver et al., 2015). After all sequence quality control, several experimental units were dropped from downstream analyses as too few sequences were retained, this was particularly true for litter samples, where we had poor DNA extraction success. Sample numbers still allowed for robust statistical analyses. OTUs were assigned taxon affinities based on the most representative sequence of the OTU (centroid). OTU richness (S_{obs}), Diversity (Compliment of Simpson's diversity index; 1-*D*), Evenness (Simpson's Evenness, E_D) (Simpson, 1949), and Bray-Curtis dissimilarity values (Bray and Curtis, 1957) were calculated for each dataset by implementing an iterative subsampling approach (1000 iterations). Each experimental unit was queried at a subsampling depth of 10,000 sequences per sample, and the average values used for downstream analyses.

2.9. Statistical analysis

To investigate if diversity estimates of millipede associated microbes change with our experimental framework, a series of repeated measures analyses (MANOVA framework), whereby matching diversity estimates for each microbial group (Fungi or Bacteria) for each timepoint were tested to see they differ across sampling time (Day 10 vs Day 30), substrate (feces, soil, or litter), nitrogen addition levels (ambient, +10 kg/ha, +20 kg/ha), all whilst accounting for a repeated measure (time) effort. To examine if microbial diversity estimates change in soil with millipede presence, similar repeated measures analyses were conducted for fungal and bacterial data against time, millipede occurrence (present, absent), nitrogen addition levels (ambient, +10 kg/ha, +20 kg/ha), controlled for repeatedly measures (time) samples. Further, to confirm nitrogen additions elicited different nitrate levels in soils, we tested soil nitrate levels (see section 2.4) using a similar repeated measures analysis. Repeated measures MANOVAs were conducted (with JMP Pro v14; SAS Institute; Cary, NC, USA) with transformed data to increase normality of data where needed. For both objectives, Diversity and Evenness data were transformed using logit functions and richness data were transformed using Box-Cox transformations (λ=2). Where models were significant, appropriate post-hoc (Student's t-test or Tukey HSD) tests were conducted.

To investigate if the experimental treatments elicited a shift in microbial communities, we conducted a series of PerMANOVA (Anderson, 2001) tests (function adonis in the package *vegan* (Oksanen et al., 2017)) using R (v.3.3.3; R Core Team, 2017). Each PerMANOVA was conducted using 999 permutations to calculate Pseudo-F statistics. For the soils, our aim was to investigate impacts of millipede occurrence on soil communities, thus, our PerMANOVA model included millipede occurrence (present, absent), nitrogen addition levels (ambient, +10 kg/ha, +20 kg/ha), time (Day 10, Day 30), and all possible interactions. For the millipede fecal analysis, we aimed to investigate if the millipede microbiome (fecal) is directly impacted by nitrogen levels and sampling day (Day 10, Day 30). Given that our initial PerMANOVA of substrate (litter, soil, feces) indicated strong impacts of substrate for both bacteria ($F_{2,53} = 34.88$, $P = 0.001$, $R^2 = 0.568$) and fungi ($F_{2,62} = 19.16$, $P = 0.001$, $R^2 = 0.382$), we constrained additional PerMANOVA models to only fecal samples and includes nitrogen amendment levels, Day, and N $\times$ Day interactions. Communities were visualized using non-metric multidimensional scaling (NMDS as implemented in mothur; 1000 instructions using Bray-Curtis dissimilarity values).

Further, since there is a paucity of information on what microbes make up the millipede gut microbiome, we wished to interrogate this system in more detail. We investigated phyla and family level shifts in taxonomic relative abundances for fungi and bacteria with N-amendments (ambient, +10 kg/ha, +20 kg/ha), Substrate (soil, litter, fecus), Day (Day 10, Day 30), and all possible interactions using a fully factorial three-way Zero-Inflated Negative Binomial (ZINB) generalized regression framework. ZINB was used as used opposed to other zero-inflated models (ZI Poisson and ZI Gamma) after model selection where ZINB has lower AICc values. Relative abundances for phyla and families were tabulated where OTUs could be resolved with >90% bootstrap support. Where significant, appropriate post-hoc tests were conducted.

To examine individual OTU responses, we conducted Linear Discriminant Analysis (LDA) effect size (LEfSe) analysis as implemented in mothur (Segata et al., 2011). For the soils, we aimed to identify biomarker OTUs that are overrepresented with millipede occurrence (class) whilst accounting for N levels (subclass). This resulted in identification of OTUs that are strongly determined by either millipede presence or absence in the soil. For the millipede microbiome objective, we identified fungal and bacterial OTUs that are biomarkers for substrates (soil, litter, or feces; class) whilst accounting for N levels (subclass). To further elucidate putative ecological function of biomarker taxa, we used a combination of the fungal functional database FungalTraits (v.1.2; Põlme et al., 2020) and primary literature for bacterial biomarkers based on their identities (Cavaletti et al., 2006; Rosenberg, 2014; Yang et al., 2005).

We investigated if *C. g. georgiana* gut microbial communities (fecal material proxies) are more likely than chance to be a subset of paired soil or litter communities. To test if fecal communities were deterministically, versus independently stochastically, structured from soil or litter communities within the same mesocosm for each day samples (Day 10, Day 30), we calculated pairwise β_{RC} (Beta Raup-Crick) values (Chase et al., 2011; Raup and Crick, 1979) to estimate the probability of deterministic community assembly (*following* Brown et al., 2020) of fecal material as compared to null models based on data randomization. Using the program PaST (v3.12; Hammer et al., 2001) with 1000 replicates, we calculated pairwise standardized β_{RC} values between feces and soil or feces and litter paired samples (same mesocosm; $-1 \leq \beta_{RC} \leq 1$, where $|\beta_{RC}| > 0.95$ indicates divergence from the null expectation; two-tailed test, alpha = 0.05). It is reasonable to assume that fecal communities are a mixture of soil and litter communities, thus we compared β_{RC} values (when approaching a value of 1, the more similar these compared communities are as compared to chance) using a combination of two-tailed Wilcoxon Signed-Rank tests and Van der Waerden two-tailed exact tests (Van der Waerden, 1952) on β_{RC} values to determine whether community similarity between fecal and soil or litter differed across days.

All statistics were conducted using a combination of JMP Pro, R, mothur, Excel, and PaST 3.

3. Results

3.1. Nitrogen measurements

Nitrogen amendments were seen to have had the intended effect on mesocosm nitrate levels with (Ambient < +10 kg/ha < +20 kg/ha) throughout our experiment ($F_{2,54} = 37.277$, $P < 0.001$) based on repeated measures. Nitrate did not differ between the millipede and no millipede treatments ($F_{1,54} = 0.123$, $P = 0.728$).

3.2. Sequence information

After all quality control methods, total retained sequences and demarcated OTUs for the soil analysis were: Bacteria - ~1.9 million sequences and 3321 OTUs; Fungi - ~3.2 million sequences and 752 OTUs. For the millipede microbiome analysis, we retained: Bacteria - ~2.2 million sequences and 4532 OTUs; Fungi - ~3.4 million sequences with 713 OTUs. All sequences are deposited in the Sequence Read Archive (SRA) and NCBI under the following accessions: BioProject (PRJNA713762) and BioSamples (Fungi: SAMN18260586-SAMN18260701; Bacteria: SAMN18260932-SAMN18261047).

3.3. Taxonomic summary

Overall, microbial communities were diverse and complex and bacterial libraries were dominated by the phyla Proteobacteria and Acidobacteria. Abundant bacterial families include the Burkholderiaceae and Planctomycetaceae with the Chitinophagaceae also being heavily represented. Fungi were dominated by Ascomycota and Basidiomycota with heavy representation of the 'Zygomycota'. Abundant families include the Trichocomaceae, Herpotrichiellaceae and the Mortierellaceae. Overall, there were numerous OTUs within the genera *Burkholderia*, *Gaiella, Mucilaginibacter*, and *Gemmata* within bacteria and *Penicillium, Mortierella, Cladophialophora, and Talaromyces* within fungi - see Table S2 for a taxonomic summary and Table S3 for full taxonomic identities. It is important to note that fungal taxa identified as 'Zygomycota' actually belong to the recently delineated Phylum Mucoromycota (Spatafora et al., 2016) which restructured the 'Zygomycota' including absorbing the formal phylum Glomeromycota. However, this reorganization is incomplete and the UNITE reference taxonomic

dataset used does not yet incorporate these lineage rank changes. However, for our observed taxa, best taxonomic identities contained no Glomeromycotina, and mainly consisted of Mucorales, and Mortierellomycotina species (Table S3). Consequently, we use the terminology Zygomycota *sensu latissimo*.

3.4. Diversity estimators and community shifts

For Objective 1 (soils), diversity estimates of fungi did not differ with our experimental framework (S_{obs} – $F_{5,19} = 0.56$, P = 0.668; 1-*D* – $F_{5,19} = 0.984$, P = 0.00.453; E_D – $F_{5,19} = 0.915$, P = 0.492), nor did bacterial diversity estimates (S_{obs} – $F_{5,15} = 1.259$, P = 0.331; 1-*D* – $F_{5,15} = 0.869$, P = 0.530; E_D – $F_{5,15} = 1.319$, P = 0.308; see Table S4 for full test statistics). Bacterial diversity estimates (S_{obs}, 1-*D*, and E_D) were seen to decrease with time (Day 10 > Day 30; Table S4).

For Objective 2 (millipede microbiome), richness and diversity estimates were generally responsive for fungi (S_{obs} - $F_{8,18} = 4.293$, P = 0.005; 1-*D* - $F_{8,18} = 3.165$, P = 0.201) and bacteria (S_{obs} - $F_{8,18} = 3.532$, P = 0.013; 1-*D* - $F_{8,18} = 2.226$, P = 0.072), but evenness did not change across our model for either bacteria (P = 0.293) or fungi (P = 0.364) This was driven mainly by substrate effects with both richness and diversity having higher values in soil (Soil > Fecal > Litter) for both fungi and bacteria. Further, bacterial richness was lower with increased N addition (ambient ≥ +10 kg/ha > +20 kg/ha) and fungal richness decreased with day and day within litter.

PerMANOVA tests indicate that soil communities are different when millipedes are present (Table 1; Fig. 1; Fungi - $F_{1,46} = 3.012$, P = 0.012; Bacteria - $F_{1,36} = 2.379$, P = 0.017). Further, both fungal and bacterial communities shift across days and nitrogen amendments alter bacteria communities. Since substrate played the major role in structuring communities in the millipede microbiome experiment for bacteria ($F_{2,53} = 34.88$, P = 0.001, $R^2 = 0.568$) and fungi ($F_{2,62} = 19.16$, P = 0.001, $R^2 = 0.382$), further examination of whether the millipede microbiome (fecal) was structured by N-amendments, day, and/or their interactions was conducted. Fecal communities shifted with sampling day for both fungi ($F_{1,19} = 2.96$, P = 0.009) and bacteria ($F_{1,18} = 7.377$, P = 0.001) but not nitrogen amendment levels or their interactions (Table 1).

Table 1
Results of PerMANOVA tests for the soil experiment and millipede microbiome experiment testing if communities differ with factors (millipede – presence or absence, N – nitrogen amendment levels, and day – day 10 vs day 30) and associated interactions. Presented are Pseudo-F test statistics, R^2 values, and p-values. Significant factors are in bold and italicized. N is nitrogen amendment treatments.

Soil Experiment			
Test	Pseudo-F_{df}	R^2	P-value
Fungi			
Millipede	***$F_{1,46} = 3.012$***	***0.047***	***0.012***
N	$F_{2,46} = 1.288$	0.041	0.186
Day	***$F_{1,46} = 7.874$***	***0.125***	***0.001***
Millipede x Day	$F_{1,46} = 1.188$	0.018	0.259
Millipede x N	$F_{2,546} = 1.271$	0.040	0.201
Residuals		0.727	
Bacteria			
Millipede	***$F_{1,36} = 2.279$***	***0.045***	***0.017***
N	***$F_{2,36} = 1.856$***	***0.071***	***0.025***
Day	***$F_{1,36} = 6.675$***	***0.127***	***0.001***
Millipede x Day	$F_{1,36} = 1.184$	0.022	0.268
Millipede x N	$F_{2,36} = 1.145$	0.043	0.277
Residuals		0.689	
Millipede Microbiome – Fecal Samples			
Test	**Pseudo-F_{df}**	**R^2**	**P-value**
Fungi			
N	$F_{2,19} = 0.873$	0.071	0.579
Day	***$F_{1,19} = 2.967$***	***0.121***	***0.009***
N x Day	$F_{2,19} = 0.399$	0.326	0.991
Residuals		0.775	
Bacteria			
N	$F_{2,18} = 0.941$	0.061	0.473
Day	***$F_{1,18} = 7.377$***	***0.238***	***0.001***
N x Day	$F_{2,18} = 1.844$	0.119	0.065
Residuals		0.582	

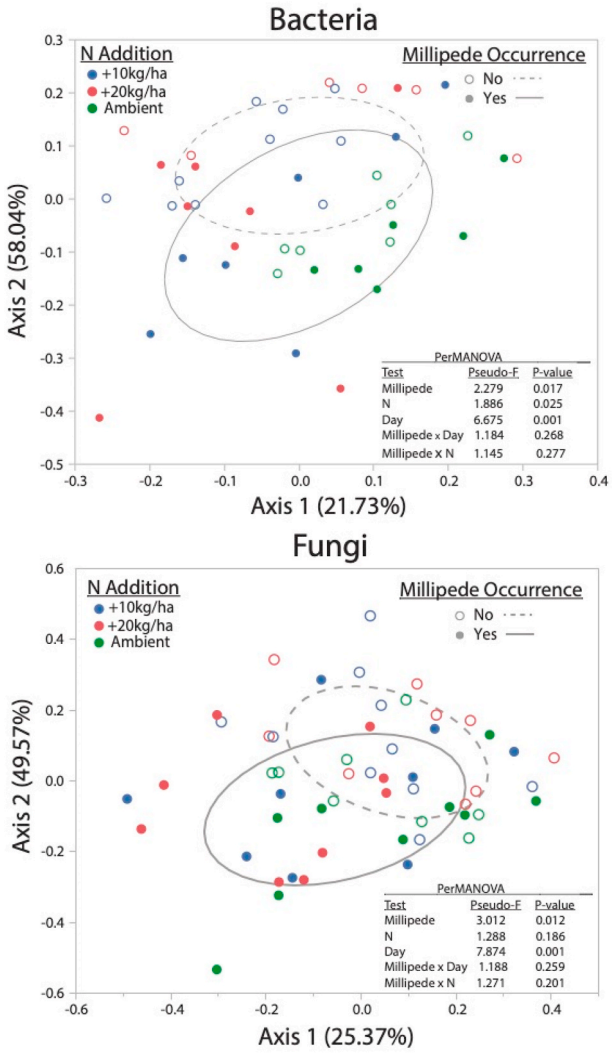

Fig. 1. NMDS ordinations of soil communities for bacteria (top) and fungi (bottom) coded with nitrogen amendment levels (color coded), millipede occurrence (closed or open circles) for the two most explanatory axes. Also presented are PerMANOVA results and 50% density ellipses for millipede occurrence. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Of the 17 delineated bacterial phyla within the millipede microbiome analysis, only one (candidate phylum Latescibacteria, previously WS3) did not have significant changes in relative abundances across any tested factors (fully factorial three-way ZINB regression with full interactions - N-amendments, Substrate, and Day; see Table S5 for full test statistics). Abundant phyla generally have differential abundances across substrates (Proteobacteria - Litter > Feces > Soil; Planctomycetes - Soil = Feces > Litter; and Acidobacteria - Soil > Feces = Litter) or nitrogen additions (Acidobacteria - ambient = +10 kg/ha>+20 kg/ha, and Actinobacteria - 10 kg/ha = +20 kg/ha > ambient). Further, of the 103 demarcated bacterial families, 77 demonstrated significant changes in relative abundance across at least on factors of our model (see Table S5). Of the five fungal phyla tested, the relative abundances of all five phyla were significantly affected by one or more factor in our model (Table S5) with Ascomycota differing with substrate (Litter > Soil =

Feces); Basidiomycota differing across substrates (Feces > Soil > Litter), Day (Day 10 > Day 30), N x Day, Substrate x Day (but unchanging in fecal pellets), and N x Day x Substrate (but unchanging in fecal material); and 'Zygomycota' which differed across substrate (Soil = Feces > Litter). Of the 74 resolved fungal families, 62 were significantly different in relative abundances across at least one of our factors tested (Table S5).

3.5. Biomarker taxa

We identified biomarker OTUs that are significantly overrepresented in soils with and without millipedes or in fecal samples all whilst controlling for nitrogen treatment effects (coded as subclass) using LEfSe. For soils, 99.4% of the bacterial OTUs and 97.1% of the fungal OTUs tested were equally abundant in soils with and without millipede occurrence, but several OTUs were seen to be biomarker taxa of millipede presence (18 bacterial OTUs and 22 fungal OTU) several of which were abundant (within the 100 most abundant OTUs) in our system (Table 2). Further, 189 bacterial OTUs (4.2%) and 45 fungal OTUs (6.3%) were considered biomarkers for millipede fecal material, several of which were highly abundant (Table S6). Biomarkers for fecal material were diverse. The fungal biomarkers were dominated by OTUs best assigned to the families Trichocomaceae [Ascomycota; Eurtiomycetes] (17.7%), which includes members within the saprotrophic genera *Penicillium*, *Aspergillus*, and *Paecilomyces*, Herpotrichiellaceae [Ascomycota; Eurtiomycetes] (11.1%), all within the saprobic genus *Cladophialophora*, and Umbelopsidaceae [Mucormycota; Umbelopsidomycetes] (8.8%), all within the saprobic genus *Umbelopsis*. The bacterial biomarkers were dominated by OTUs best assigned to the families Chitinophagaceae, which are presumed chitin degraders (Rosenberg, 2014) [Bacteroidetes; Sphingobacteriia] (11.1%), which primarily consisted of unclassified genera but also *Flavisolibacter*, *Parafilimonas*, and *Taibaiella*, Burkholderiaceae [Proteobacteria; Betaproteobacteria] (5.8%), a family with broad ecologies (Coenye, 2014) consisting of members of the genera *Burkholderia* (diverse C and N utilization) and *Cupriavidus* (chemoheterotrophic or chemolithotrophic), and Porphyromonadaceae [Bacteroidetes; Bacteroidia] (5.8%), consisting of *Dysgonomonas* sp. (often found in fecal samples and may produce lactic acid; Sakamoto, 2014) and an unclassified Porphyromonadaceae. This suggests a diverse suite of microbes can preferentially colonize millipede digestive tracts and/or exploit millipedes.

Table 2
Abundant significant Biomarkers (LEfSe) in soil that are enriched in with the presence or absence of millipeds along with P-values, best taxonomic identities and putative ecological roles for Bacteria and Fungi.

OTU ID	Enriched in Presence or Absence of Millipede?	P-Value	Taxonomy	Ecological Role
Bacteria				
Otu00046	Presence	0.030	Chitinophagaceae sp.	Unknown (Potential Chitin Degrader)
Otu00055	Presence	<0.001	Chitinophagaceae sp.	Unknown (Potential Chitin Degrader)
Otu00069	Presence	0.016	*Silvimonas* sp.	Putative Chitin Degrader
Otu00081	Presence	0.009	Chitinophagaceae sp.	Unknown (Potential Chitin Degrader)
Otu00085	Absence	0.004	*Actinospica* sp.	Acidophilic
Otu00094	Presence	0.001	Enterobacteriaceae sp.	Unknown
Fungi				
Otu00007	Presence	<0.001	*Trichosporon gamsii*	Probable Animal Pathogen
Otu00015	Presence	<0.001	Ascomycota unclassified	Unknown
Otu00022	Absence	0.015	*Meliniomyces*_sp	Dark Septate Endophyte
Otu00026	Absence	0.027	*Pseudogymnoascus roseus*	Soil Saprobe
Otu00029	Presence	<0.001	Sporidiobolales sp	Unknown
Otu00063	Presence	<0.001	*Candida boleticola*	Probable Animal Pathogen
Otu00081	Presence	<0.001	*Ophiostoma aurorae*	Plant Pathogen (Insect Reservoir)
Otu00082	Presence	0.021	Helotiales sp	Unknown
Otu00083	Absence	0.020	*Umbelopsis ovata*	Saprobe

3.6. Millipede gut community origins

Investigations into whether fecal communities within our millipedes were more similar to soil or litter communities (β_{RC}) indicate that fecal communities are highly similar and deterministically structured by both soil and litter for both Day 10 and Day 30. Overall, Bacteria had very high β_{RC} values but β_{RC} values were not different between soil and litter comparisons for either Day 10 (soil median = 1.0, litter median = 0.9885, Wilcoxon S = 180, P = 0.8265; Van der Waerden S = 0.031, P = 0.9886) or Day 30 (soil median = 1.0, litter median = 1.0, Wilcoxon S = 155, P = 0.7145; Van der Waerden S = 0.665, P = 0.7267). For fungi, we see that fecal communities are more similar to soil communities in Day 10 (soil median = 1.0, litter median = 0.757, Wilcoxon S = 226.5, P = 0.031; Van der Waerden S = 4.792, P = 0.035), but not Day 30 (soil median = 1.0, litter median = 1.0, Wilcoxon S = 156, P = 0.125; Van der Waerden S = −2.664, P = 0.167). This suggests fungi, but not necessarily bacteria, are more likely to be derived from soils than from litter, potentially due to a stronger environmental filtering mechanism.

4. Discussion

Here, we present an investigation into millipede effects on soil microbial communities and on the interrelation between nitrogen amendments and millipede occurrence on soil communities using next-generation sequencing. Further, we present the first NGS-based investigation into the millipede gut microbiome (but see non peer reviewed pre-print by Geli-Cruz et al., 2019) and investigate if millipede fecal community structure is impacted by increased environmental nitrogen loads. In doing so, we demonstrate novel understandings of millipede-soil interactions and impacts on nitrogen levels on soil-millipede and soil-millipede-litter fecal community interactions toward a better understanding of 1) how an increasingly nitrified world might impact soil and millipede microbial communities, 2) how millipedes help shape soil microbial communities, and 3) the colonization dynamics of millipede gut communities. It is striking that these observed differences in microbial community and taxa responses occurred, in many instances, so quickly. This is partly because bacteria and yeasts (or yeast-like fungi) are known to respond rapidly to changing environments (Lau and Lennon, 2012; Nguyen et al., 2020), but it might be possible that the mesocosm approach used here facilitated the expediency of these interactions due to strict habitat boundary limitations.

4.1. Community responses

Millipedes generally minimally impact the distributions of soil aggregates (Snyder et al., 2009), and microbial communities are known to differ across such aggregates (Bach et al., 2018), so it is perhaps surprising that millipede occurrence significantly shifts fungal and bacterial communities in soils, albeit with relatively low coefficients of

determinations in our PerMANOVA tests (Table 1). However, the millipedes in this experiment were significantly larger than those in the Snyder et al. (2009) experiment, and the mesocosms were significantly smaller. Impacts on soil communities by millipedes may be direct, but presumably have a stronger influence through indirect and/or trophic regulations (Thakur and Geisen, 2019). Millipede ecology has long been centered around indirect effects via leaf litter comminution (Hopkin and Read 1992). Importantly, these processes have not been quantified and in some cases are undefined for millipedes. The mesocosm approach employed here allows enough standardization to determine and quantify these effects. However, this approach does have limitations because the environment created may not fully recreate natural conditions (e.g., millipede density exceeds field density, other organisms are excluded, environmental conditions are stable, etc.). Further testing with other millipede species, over longer periods of time, and in more realistic field conditions will be needed to fully understand the consequences of these results.

4.2. Individual taxa responses

Abundant bacterial biomarker OTUs that belong to the Chitinophagaceae (Chitinophagaceae sp. at the genus level) are enriched in soils with millipedes present, these are potential chitin degraders (although many other ecological roles occur in this diverse family), this is in addition to a putative chitin degrader within the genus *Silvimonas* (OTU69; Yang et al., 2005) also enriched in millipede influenced soils. This strongly suggests increased chitinolytic capabilities of bacteria in soils when millipedes are present. This is likely directly associated with millipedes but could also be the result of targeted fungal degradation in millipede influenced soils as fungi are also chitinous, more work is needed to confirm functionality of these bacteria. Of the fungi that are also enriched in millipede influenced soils, they have broad action (Table 2), but several abundant enriched OTUs are worth exploring here. OTU7, which is best identified as *Trichosporon gamsii* (100% bootstrap support) and OTU63, best identified as *Candida boleticola* (100% bootstrap support) are yeast-like fungi that are probable animal pathogens (Irinyi et al., 2015). Interestingly, one of the OTUs that is enriched, OTU81 – *Ophiostoma aurorae* (93% bootstrap support for species identity, 100% bootstrap support for genus identity) is an known insect-vectored plant pathogenic microfungus usually transmitted to Pinaceae species via bark beetle transmission (Tedersoo et al., 2014; Zhou et al., 2006). Diseases caused by *Ophiostoma* includes the devastating Dutch Elm Disease. This may suggest that millipedes may represent a novel reservoir of *Ophiostoma* (*O. aurorae* was also a biomarker for millipede fecal material, LDA = 3.108, $P < 0.0001$, Table S6), which may have intriguing ecological ramifications for this devastating pathogen. However, additional work is needed to confirm that these *Ophiostoma* spp. are pathogenic on plants. Though not assessed here, millipedes may influence soil communities through synergistic interactions between millipedes and endogeic earthworms. This can occur through coprophagy of millipede droppings by the endogeic earthworms, which leads to more soil disturbances via increased earthworm biomass (Bonkowski et al., 1998), in turn influencing soil communities. However, despite large amounts of consumption of leaf litter and subsequent fecal deposition by millipedes, millipedes have surprisingly little impact on carbon mineralization rates in soils (Coulis et al., 2013). Additional examination is needed to disentangle the interactive effects of millipedes and other micro- and macroinvertebrates on soil community structure.

4.3. Presumed functional impacts

Despite the presumed role of millipedes as broad generalist consumers of soil and leaf litter (Hopkin and Read, 1992; Steinwandter and Seeber, 2020), it is unclear exactly to which extent and in which conditions most millipedes will preferentially consume soil or litter. Further confounding our understanding of millipede feeding ecology is differential foraging behavior facilitating niche separation, the details of which are only beginning to be elucidated (Semenyuk and Tiunov, 2019). Because food source remains somewhat a mystery, the origin of gut associated microbial communities in millipedes is uncertain. It has been demonstrated that fungal communities found within *Brachycybe lecontii* millipedes reared on different wood species showed some differentiation in fungal communities (Macias et al., 2019). Furthermore, enzyme extractions from millipede hindguts and midguts demonstrates cellulases and chitinases are common (Šustr et al., 2020a,b), which are likely derived from extant microbial populations. This abundance of cellulases in millipede guts is likely an important component for deriving energy from cellulose rich plant matter and may explain why coprophagy is common, as it has been suggested that some xystodesmids may undergo multiple coprophagic events to aid cellulose breakdown (McBrayer, 1973). Here, we wanted to quantify taxa that are associated with fecal material and illuminate the putative origin of the millipede gut microbiome. We identified multiple taxa that are biomarkers for fecal material (Table S6) along with multiple families that are differentially abundant across our tested substrates (soil, litter, and feces) (Table S5). Of the fungal biomarkers for feces, we see diverse lineages that are more abundant (as compared to soil and litter) including *Cryptococcus podzolicus* (OTU1), *Trichosporon gamsii* (OTU2), *Aspergillus aceleatus* (OTU17) and *Umbelopsis* sp. (OTU20) among others. Most members of the genera *Cryptococcus, Trichosporon,* and *Aspergillus* are presumed opportunistic animal pathogens (Irinyi et al., 2015). Additional work is needed to determine if these are pathogenic on millipedes. Interestingly, *Cryptococcus podzolicus* and many *Trichosporon* species are also promising oleaginous yeasts (Schulze et al., 2014) suggesting the potential for novel industrial isolation sources in millipedes. Bacterial taxa overrepresented in fecal material (Table S6) include members of the genera *Flavobacterium* (OTU4), *Bradyrhizobium* (OTU5), and numerous *Mucilaginibacter* and unclassified Chitnophagaceae sp. among others. These have broad ecologies including putative pathogens (e.g. *Flavobacterium*), N-fixers (*Bradyrhizobium* – though there is no evidence of active fixation here), and complex organic compound decomposers (*Mucilaginibacter* and Chitnophagaceae; López-Mondéjar et al., 2016). Further, we observe differential abundances on the family level with substate for many taxa (Table S5) including the fungal families Hypocreaceae (Sordariomycetes), Ophiostomataceae (Sordariomycetes), Trichomonascaceae (Saccharomycetales), and Trichosporonaceae (Tremmales) which were enriched in fecal material. Of these, the Ophiostomataceae are interesting as these are often plant pathogens (e.g., *Ophiostoma* – see above) and this gives more credence to the idea millipedes may be novel reservoirs of these plant pathogens. An additional aim of this work was to see if we can determine if millipede gut communities are primarily structured from soil or litter community origins. To this end, we use a probabilistic approach (β_{RC}) to examine if fecal communities are deterministically structed from either soil or litter (that is, is the fecal community a subset of the communities from either substrate). While we determined that both bacterial and fungal fecal communities originate from both soil and litter, fungi are more derived from soil as compared to litter. This fills a major knowledge gap in our understanding of millipede gut communities and provides a framework for additional manipulative studies to further test conserved functionality between soil and fecal communities.

4.4. Synthesis

Here, we observe numerous interactions and associations between nitrogen addition levels, substrate usage, and time (Table 1, Tables S4 and S5). How exactly millipedes are impacted by these interactions remains partially unresolved, but this suggests that in a changing world, millipedes will have to alter their food sources to adapt to shifting substrate quality. In addition, millipedes (and other animal decomposers) will have to contend with increased competition from other

decomposers including earthworms (Snyder et al., 2009), and fungi and bacteria (Scheu and Schaefer, 1998), whose populations may be increased as decomposition rates increase with temperature and nitrogen additions (Bonanomi et al., 2014). It is possible that combined stressors - including novel competition from invasive decomposers (e.g., pheretimoid earthworms, Snyder et al., 2011), may push some populations beyond their ability to cope. Accurate population size estimates in addition to an improved understanding of millipede-microbiome interactions will be critical to conserving biodiversity and functioning of the soil system.

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.

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Appendix A. Supplementary data

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