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Changes in soil fungal communities in response to invasion by *Lumbricus terrestris* Linnaeus, 1758 at Stormy Lake, Nikiski, Alaska

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by Matt Bowser[1]

Introduction

It is now well established that European earthworms (Lumbricidae) introduced to forests of North America reduce or remove preexisting leaf litter and organic layers (Bohlen et al., 2004; Hale et al., 2005; Suárez et al., 2006), altering nutrient cycles and the soil carbon balance (Bohlen et al., 2004; Hale et al., 2005; Resner et al., 2015). Exotic earthworms can also reduce the abundance of soil fungi in general (Dempsey et al., 2011) and affect mycorrhizal fungi in particular (McLean et al., 2006; Szlávecz et al., 2011), thereby impacting plants that depend on mycorrhizal relationships (Lawrence et al., 2003).

Although the consequences of exotic earthworm invasion have been demonstrated in formerly glaciated regions of North America (see Frelich et al., 2006, for a review), less is known about how earthworms might change Alaskan forests, where the climate is colder than most other locations where invasive earthworm effects have been studied. Alaskan forests are also generally at a much earlier stage of invasion than other formerly glaciated regions where earthworm populations are now well established, offering opportunities to learn more about incipient invasions.

Invasive lumbricid earthworm species can be grouped into three functional types based on their feeding and burrowing habits (Bouché, 1977). Epigeic species live at the soil surface, feeding on leaf litter. Endogeic species borrow horizontally belowground in mineral soil. Anecic earthworms excavate vertical burrows. Of these functional types, anecic species alter previously earthworm-free soils more than others (Frelich et al., 2006). By foraging on organic litter at the ground surface, feeding in vertical borrows, and depositing casts that form new mineral topsoil, anecic worms consume upper organic soil layers and vertically homogenize the soil profile through their burrowing and mixing.

In Southcentral Alaska the only anecic species known to be established is *Lumbricus terrestris* Linnaeus, 1758; other anecic species including *Aporrectodea longa* (Ude, 1895) and *Lumbricus friendi* Cognetti, 1904 have not been documented in this region. Outside of developed areas, *L. terrestris* has been found at boat launches in this region, likely introduced via "bait abandonment" (Saltmarsh et al., 2016).

Lumbricus terrestris was first documented at Stormy Lake, Nikiski Alaska in 2012 at a public boat launch (Eskelin and Bowser, 2012). Later it was observed that *L. terrestris* had already removed the leaf litter layer at this locality, exposing tree roots (Bowser, 2016*a*,*d*). Other soil-dwelling oligochaetes known to be present in this area were the exotic, epigeic earthworm *Dendrobaena octaedra* (Savigny, 1826) (Bowser, 2016*d*), which is almost ubiquitous near roads on the Kenai Peninsula (Saltmarsh et al., 2016); the native, epigeic earthworm *Bimastos rubidus* (Savigny, 1826) (Bowser, 2016*b*), and enchytraeids including *Fridericia ratzeli* (Eisen, 1872) (Bowser, 2016*c*).

In this small pilot study I sought to learn how invasion by *L. terrestris* might change the composition of soil fungal communities—especially mycorrhizal fungi—in Southcentral Alaskan forests where it has been introduced and subsequently established.

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Methods

Field Methods

At Stormy Lake I selected six sites: three within the infestation of *L. terrestris* near the boat launch and three on a transect along the boat launch access road (Figure 1). To keep the habitat as similar as possible I selected sites under cottonwoods (*Populus ×hastata* Dode), birch trees (*Betula pendula* subsp. *mandshurica* (Regel) Ashburner & McAll. or *Betula kenaica* W.H.Evans), and alders (*Alnus viridis* A.Gray).

All samples were collected on September 25, 2017. At each site I used a trowel to take a soil core from the soil surface to 6 cm depth, enough to fill a 90 ml urine sample cup. Earthworm casts, where present, were included in and represented a small portion of the soil samples. Field notes are provided in Bowser (2018).

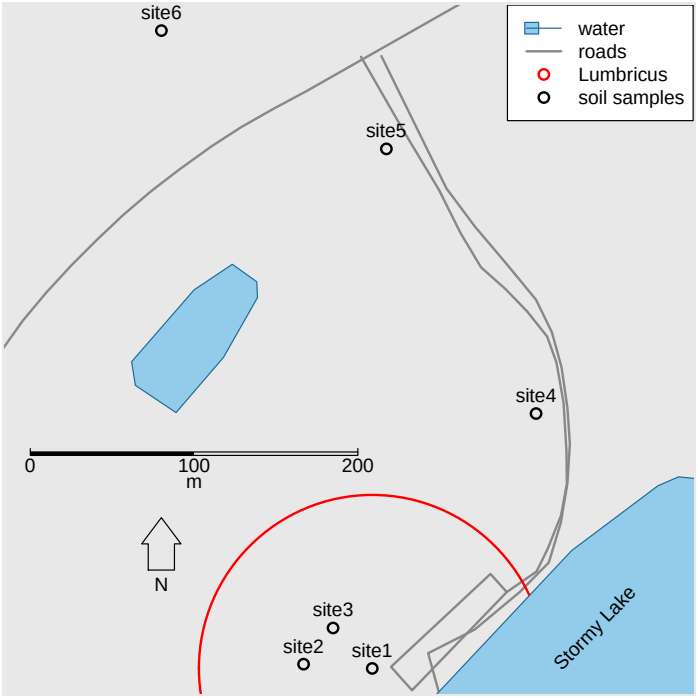

Figure 1: Map of sampling locations with the approximate limit of the *Lumbricus terrestris* infestation.

Laboratory Methods

Soil samples were stored briefly in a -22° C freezer, then shipped to RTL Genomics (RTL) in Lubbock, Texas (`http://rtlgenomics.com/`), where they were submitted for RTL Genomics' Microbial Diversity Assay with a target of an average of at least 10,000 reads per sample.

RTL Genomics performed DNA extractions using a Qiagen MagAttract PowerSoil DNA KF Kit. For soil samples, each whole sample was agitated and a pea-sized amount was added to the Qiagen MagAttract PowerSoil DNA KF Kit bead plate. 400 µl of RNase A solution was combined with 75 ml of PowerMag Bead solution and 750 µl of this solution was added to each well of the KF plate. 60µl of warmed lysis buffer was added to each well. The plate was placed on a TissueLyser for 10 min. at 20 Hz. The plate was flipped and then shaken for an additional 10 min. at 20 Hz. The plate was centrifuged 10 min. at $2,700 \times g$. The supernatant was moved to a new KF plate, and 450 µl of IRT solution was added to each well. The plate was vortexed for 5 s and incubated for 10 min. at 4 °C. The plate was centrifuged for 10 min. at $2,700 \times g$. The supernatant was transferred to a new plate and centrifuged 10 min. at $2,700 \times g$. 450 µl of supernatant was moved to a new plate. 2 ml of resuspended ClearMag Beads were combined with 45 ml of ClearMag Binding Solution and mixed well. 470 µl of ClearMag Beads/Clear Mag Binding Solution was added to each well. The plate was placed on a KingFisher with 3 ClearMag Wash Plates. DNA was eluted into 100 µl EB Solution and stored at -20 °C.

Samples were amplified for sequencing in a two-step process using the ITS3F (GCATCGATGAAGAACGCAGC) and ITS4R (TCCTCCGCTTATTGATATGC) primer pair (White et al., 1990; RTL Genomics, 2017), amplifying part of the ITS2 region. The forward primer was constructed with the illumina i5 sequencing primer (5'-3': TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and the ITS3F primer. The reverse primer was constructed with the illumina i7 sequencing primer (5'-3': GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) and the ITS4R primer.

Amplifications were performed in 25 µl reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, California), 1 µl of each 5 µM primer, and 1 µl of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, California) under the following thermal profile: 95 °C for 5 min. then 35 cycles of 94 °C for 30 s, 54 °C for 40 s, 72 °C for 1 min. followed by one cycle of 72 °C for 10 min. and a 4 °C hold.

Products from the first stage amplification were added to a second PCR based on qualitatively determined concentrations. Primers for the second PCR were designed based on the illumina Nextera PCR (Forward: AATGATACGGCGACCACCGAGATCTACAC[i5index]TCGTCGGCAGCGTC, Reverse: CAAGCAGAAGACGGCATACGAGAT[i7index]GTCTCGTGGGCTCGG). The second stage amplification was run the same as the first stage except for 10 cycles.

Amplification products were visualized with eGels (Life Technologies, Grand Island, New York). Products were then pooled equimolar and each pool was size selected in two rounds using Agencourt AMPure XP (BeckmanCoulter, Indianapolis, Indiana) in a 0.75 ratio for both rounds. Size selected pools were then quantified using the Quibit 2.0 fluorometer (Life Technologies) and loaded on

an illumina MiSeq (illumina, Inc. San Diego, California) 2×300 flow cell at 10 pM.

Metagenomic Analysis

A metagenomic analysis was performed on the USGS Yeti supercomputer (`https://www.usgs.gov/core-science-systems/sas/arc`) running Scientific Linux 6.7 (`https://www.scientificlinux.org/`). Raw FASTQ files were processed using the PIPITS pipeline (Gweon et al., 2015), which has been shown to perform well for fungal metabarcoding (Anslan et al., 2018). In the PIPITS pipeline the UNITE database version 7.2 (Kõljalg et al., 2013) was used as a reference library.

The Operational Taxonomic Unit (OTU) table was filtered using the LULU algorithm (Frøslev et al., 2017) using the same settings as Anslan et al. (2018) (`minimum_ratio_type = "min", minimum_match = 97`). OTUs with less than ten sequences per sample and OTUs identified as not belonging to the kingdrom Fungi were also removed.

Fungal Community Data Exploration

To examine fungal communities by guilds, the OTU table was submitted to FUNGuild (Nguyen et al., 2016) at `http://www.stbates.org/guilds/app.php` for guild assignments. The resulting guild assignments were regrouped into a six broad groups (endophyte or parasite; endophyte, mycorrhizal, parasite, or saprotroph; endophyte, parasite, or saprotroph; mycorrhizal; mycorrhizal or saprotroph; saprotroph; and unknown). Unidentified OTUs and OTUs for which FUNGuild did not assign a guild were lumped into the unknown guild category.

Correspondence analyses was performed using the `vegan` package version 2.5-3 (Oksanen et al., 2018) in R version 3.5.1 (R Core Team, 2018). For correspondence analyses, OTU abundances in terms of numbers of reads per sample were $\log + 1$ transformed. Two types of correspondence analyses were run. In a vector fitting analysis the occurrence data were processed by the `cca` function, then the presence of *L. terrestris* was fitted as an environmental factor using the `envfit` function. In a constrained correspondence analysis, the occurrence data were fitted with the `cca` function where the presence of *L. terrestris* was included as a constraint.

Notes from the analyses are included in Bowser (2019*b*) and Bowser (2019*a*).

Data Deposition

Sequence data were deposited in GenBank Sequence Read Archive under Bioproject PRJNA525443.

Results

The metagenomic analysis yielded 48,315 reads representing 623 observations of 402 Operational Taxonomic Units (OTUs). Numbers of OTUs dectected at each site were similar between infested sites, where 95–116 (mean = 103) OTUs per sample were found and nightcrawler-free sites, where 74–139 (mean = 105) OTUs per sample were found.

Most fungal OTUs were detected in only one of the six samples (Figure 2). Nine OTUs were found at all six sites. These were identified as Mycosphaerellaceae sp. SH206770.07FU, *Mortierella humilis*, *Solicoccozyma terricola*, Polyporales sp. SH187220.07FU, Pseudeurotiaceae, Nectriaceae, *Chalara* sp. SH202710.07FU, an unidentified ascomycete, and two unidentified fungi.

Fungal community composition differed between infested and non-infested plots (Figure 3, Table 1). Mycorrhizal fungi made up 21% of reads where *Lumbricus* was absent and 6% of reads where *Lumbricus* was present. Saprotrophs and fungi with unkown ecological roles represented a larger number of reads at infested sites compared to non-infested sites.

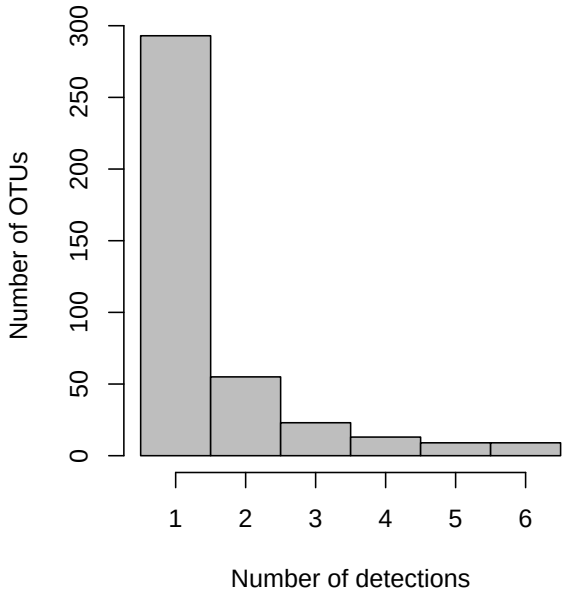

Figure 2: Frequencies of occurrence of soil fungal OTUs.

In the vector analysis, unconstrained axes CA1 and CA2 explained 24% and 21%, respectively, of the variation (Figure 4.a) The fitting of *Lumbricus* presence to the unconstrained analysis was non-significant ($p = 0.3$) and mostly aligned with axis CA1. In the constrained analysis where the presence of *Lumbricus* was included as the only constraint, the *Lumbricus* axis explained 22% of the variation while unconstrained axes CA1 and CA2 each explained 21% of remaining variation (Figure 4.b). An ANOVA of the constrained analysis found the effect of *Lumbricus* presence to be significant ($p = 0.001$).

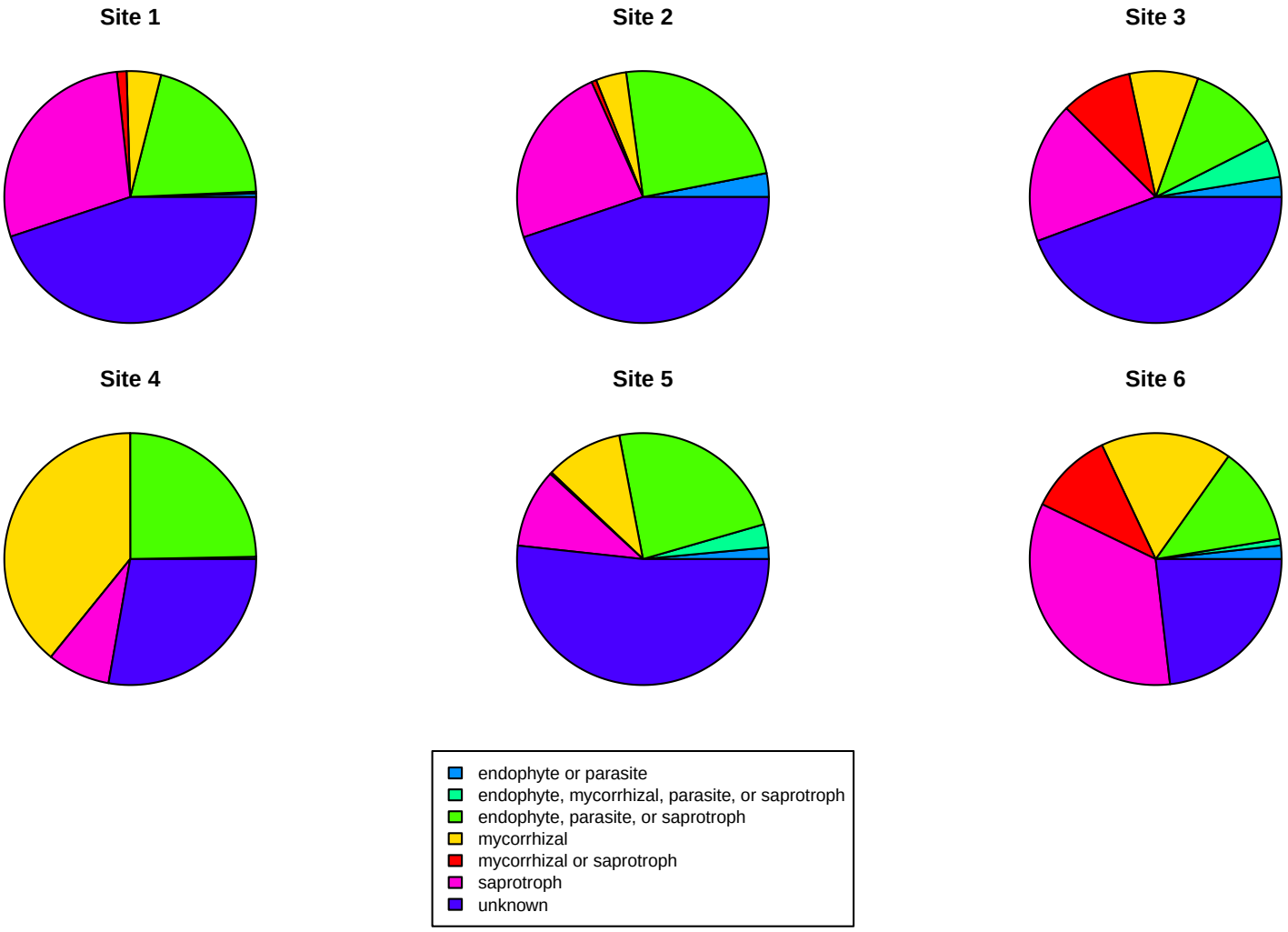

Figure 3: Proportions of numbers of reads assigned to fungal guilds represented in soil samples from the six sampled sites. *Lumbricus terrestris* was present at sites 1–3 and absent at sites 4–6.

Table 1: Numbers of reads and percentages of fungal guilds represented in soil samples from within and outside of an area infested by *Lumbricus terrestris*.

	***Lumbricus* absent**		***Lumbricus* present**	
Category	***n* reads**	**Percent**	***n* reads**	**Percent**
endophyte or parasite	298	1	467	2
endophyte, mycorrhizal, parasite, or saprotroph	354	1	487	2
endophyte, parasite, or saprotroph	5,055	21	4,254	18
mycorrhizal	5,094	21	1,443	6
mycorrhizal or saprotroph	839	3	1,021	4
saprotroph	4,137	17	5,455	23
unknown	8,822	36	10,589	45

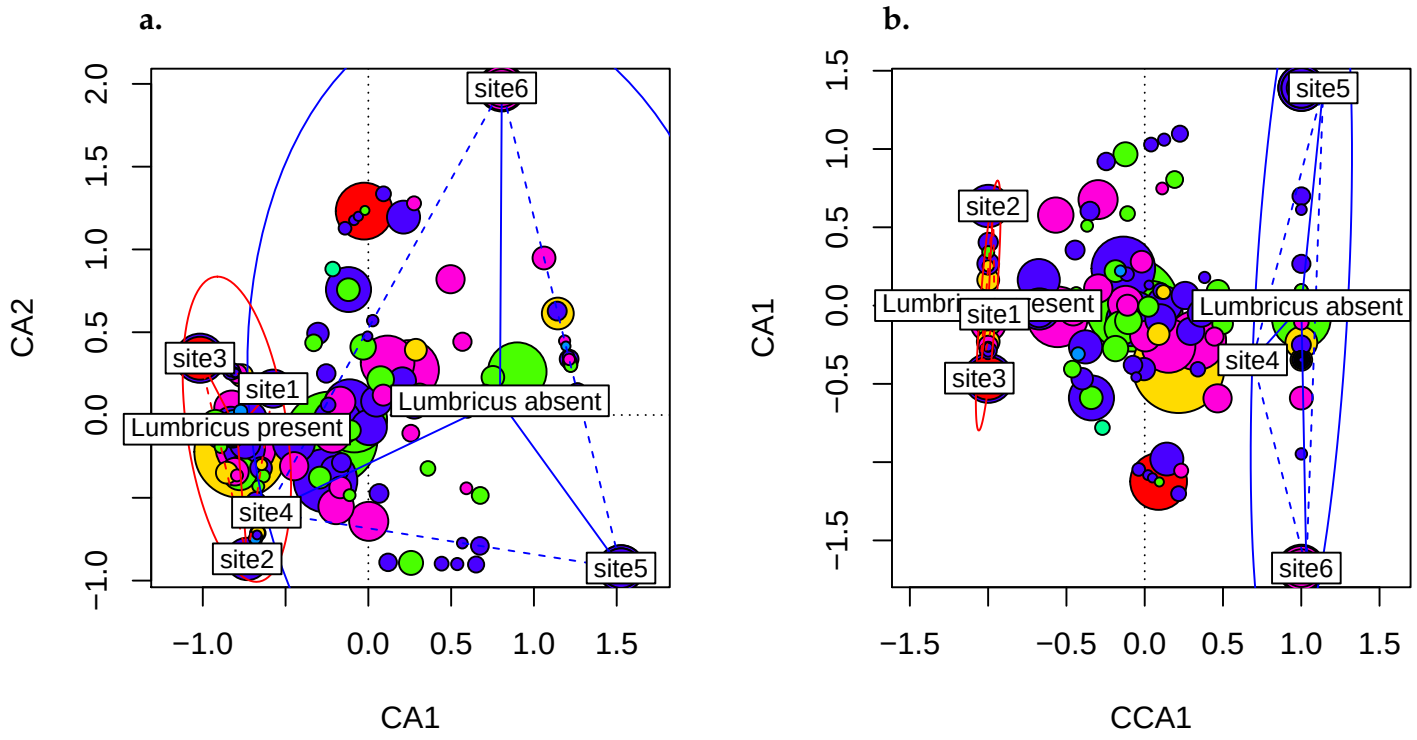

Figure 4: Biplots of OTUs and sampling sites from correspondence analyses. **a**: Vector analysis where the presence of *L. terrestris* was fitted as an environmental factor. **b**: Constrained analysis where the presence of *L. terrestris* was included as a constraint. Colors of OTU circles correspond to guilds as in Figure 3. Area of circles is proportional to total abundance of each OTU, with the smallest circles indicating 10 reads and the largest circle indicating 2,851 reads. Red and blue lines indicate groupings of sites by presence of *L. terrestris*.

Mycorrhizal species were spread almost evenly among sites with and without *Lumbricus*, but read abundances of mycorrhizal species tended to be higher in sites where *L. terrestris* was absent. The four most abundant mycorrhizal species in terms of numbers of reads were all more abundant at *Lumbricus*-free sites. These were identified using the UNITE blastn analysis (`https://unite.ut.ee/analysis.php`) as *Inocybe borealis* J.E. Lange, 1957, *Cortinarius casimiri* (Velen.) Huijsman, 1955; *Helvellosebacina helvelloides* (Schwein.) Oberw., Garnica & K. Riess, 2014; and *Oidiodendron pilicola* Kobayasi, 1969.

Discussion

Effects of *L. terrestris* on Communities

Despite a small sample size of only six sites, it appeared that invasion by *L. terrestris* did alter soil fungal communities at Stormy Lake, decreasing the proportion of the fungal community known to be mycorrhizal. These few data points add to a growing body of literature documenting the effects of exotic earthworms, especially the anecic *Lumbricus terrestris*.

Our results were consistent with the findings of McLean et al. (2006) and Szlávecz et al. (2011) that exotic earthworms decrease the abundance of mycorrhizal fungi. The response of plant symbionts to this reduction in mycorrhizal fungi varies by species (Szlávecz et al., 2011); but consequences for even one plant species can be difficult to predict (see Lawrence et al., 2003).

It should be noted that none of the sites sampled represented native soil communities because the exotic, epigeic earthworm *Dendrobaena octaedra* was present over the entire study area and that even this epigeic species has been shown to alter soil fungal communities (McLean and Parkinson, 2000).

Methodological Issues

At least some of the observed differences between fungal communities where *L. terrestris* was present or absent had to do with the fact that the leaf litter and decomposing organic matter had been all but removed in the three sites infested by *L. terrestris* while these layers were intact and included in soil cores from sites free of *L. terrestris*. Others have noted difficulties of comparing earthworm-free soils with earthworm-worked soils where the soil properties

have been greatly altered by earthworms (e.g., Lawrence et al., 2003).

I recognize that the observed differences in fungal communities may have been due to habitat differences along the transect and not due to *L. terresris*. The best way to separate the effects of *L. terrestris* would be experimental methods where earthworms could be added to or removed from experimental plots so that other sources of variation could be controlled.

In future studies it would be better to take additional relevant measurements at each site including litter depth, other soil characteristics, and the species composition, density, and biomass of earthworms. These measurements could then be included in community analyses to help understand how important the contribution of *L. terrestris* is to determining soil fungal community composition. Alternative fungal primer sets should also be considered, for example the ITS86F/ITS4 primer pair (Op De Beeck et al., 2014).

Conclusions

This small pilot study provided some evidence that invasion by *Lumbricus terrestris* is altering fungal communities where it is established in Southcentral Alaska. Appropriate next steps would be to better characterize these changes by using a more robust sampling design, to document in detail how fungal communities differ in soil profiles, and to elucidate how changes in soil fungal communities affect local plant species.

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