Title: Ecological patterns are robust to use of exact sequence variants versus operational taxonomic units

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Abstract:

Recent controversy focuses on the best method for delineating microbial taxa, based on either traditional operational taxonomic units (OTUs) or exact sequence variants (ESVs) of marker gene sequences. We sought to test if the binning approach (ESVs versus OTUs) affected the ecological conclusions of a large field study. The dataset included sequences of both bacteria (16S) and fungi (ITS), across multiple environments diverging markedly in abiotic conditions, over three collection times. Despite quantitative differences in microbial richness, we found that all alpha- and beta-diversity metrics were highly positively correlated ($r > 0.90$) between samples analyzed with both approaches. Moreover, the community composition of the dominant taxa did not vary between approaches. Consequently, statistical inferences were nearly indistinguishable. Thus, we conclude that for typical alpha- and beta-diversity analyses, OTU or ESV methods will likely reveal similar ecological results and determining which method to employ will depend on the question at hand.

Main:

Characterization of microbial communities by amplicon sequencing introduces biases and errors at every step. Hence, choices concerning all aspects of molecular processing from DNA extraction method (Frostegard et al 1999) to sequencing platform (Claesson et al 2010) are contested. Further downstream, the choices for computational processing of amplicon sequences are similarly deliberated (e.g. (Caporaso et al 2010, Edgar 2013, Schloss et al 2009)). Yet despite these ongoing debates, microbial ecology has made great strides towards characterizing and testing hypotheses in environmental and host-associated microbiomes (e.g. (Delgado-Baquerizo et al 2018, Thompson et al 2017)).

Within microbiome studies, operational taxonomic units (OTUs) have been used to delineate microbial taxa, as microbial diversity still vastly outstrips our global databases (Moyer et al 1994). While any degree of sequence similarity could be used to denote individual taxa, a 97% sequence similarity cutoff became standard within microbial community analyses. This cutoff attempted to balance previous standards for defining microbial species (Stackebrandt and Goebel 1994) and a recognition of spurious diversity accumulated through PCR and sequencing errors (Acinas et al 2005, Kunin et al 2010).

Recently, controversy over classifying microbial taxa has been renewed with the suggestion that taxa should be defined based on exact nucleotide sequences of marker genes. Delineating taxa by exact sequence variants (ESVs), also termed amplicon sequence variants (ASVs)(Callahan et al 2017) or zOTUs (Edgar 2016), is not only expected to increase taxonomic resolution, but could also simplify comparisons across studies by eliminating the need for re-binning taxa when datasets are merged. Due to these advantages, there has been a surge in

bioinformatic pipelines that seek to utilize ESVs and minimize specious sequence diversity (Amir et al 2017, Callahan et al 2016, Edgar 2016). Moreover, some proponents have stated that ESVs should replace OTUs altogether (Callahan et al 2017), and some journal reviewers are insisting that ESVs be used over OTUs as a condition for a study's publication. However, there are clear advantages to OTU classifications as they can be biologically useful for comparing diversity across large datasets (Delgado-Baquerizo et al 2018) or identifying clades that share traits (Martiny et al 2009). It is also clear that both 97% OTUs and ESVs mask ecologically important trait variation of individual taxa (Chase et al 2017, Eren et al 2015, Needham et al 2017). Yet, what remains unclear is whether binning approach affects broad ecological outcomes, and if outcomes vary based on amplicon targeted.

Thus, our focus here was to test if use of ESVs versus 97% OTUs affects broad ecological conclusions at the community level, including determining significant treatment effects and overall diversity patterns. To do so, we used a dataset from a large leaf litter decomposition field study. This study included a "site" and "inoculum" treatment, in which all microbial communities were reciprocally transplanted into all five sites (see SI Methods) along an elevation gradient (Baker and Allison 2017). We sequenced both bacteria (16S) and fungi (ITS2) from litterbags collected at three time points (6, 12, and 18 months after deployment) in separate sequencing runs. While we expected that the binning approach would alter observed richness, we hypothesized that it might not alter trends in alpha- and beta-diversity.

In total, we analyzed >15 million bacterial and >20 million fungal sequences using UPARSE v10 (**Table S1**), which allowed for a direct comparison of OTU versus ESV approaches by keeping all other aspects of quality filtering and merging consistent (Edgar 2013). ESV and OTU alpha-diversity was strongly correlated across samples using four metrics for both bacteria and fungi (mean Pearson $r = 0.95 \pm 0.02$; all P values < 0.001). For the three metrics (Berger-Parker, Shannon, Simpson), the ESV and OTU approaches were not only highly correlated (mean Pearson $r = 0.95 \pm 0.02$), but nearly equivalent in their values (mean slope = 0.97; **Table S2**). For observed richness, ESV versus OTU was also highly correlated across all time points/sequencing runs (Pearson $r > 0.92$; **Figure 1A,B**). However, bacterial OTU richness was approximately half that of ESV richness for the same sample (mean slope = 0.46), and fungal OTU richness was approximately three quarters that of ESV richness (mean slope = 0.79). We speculate that this difference between bacteria and fungi is due to the coarser phylogenetic breadth of the 16S versus ITS genetic regions.

Beta-diversity metrics were also strongly correlated across samples for ESVs and OTUs (Bray-Curtis average Mantel $r = 0.96$ for bacteria and 0.98 for fungi; all $P < 0.01$; **Figure 1C,D**), whether assessed by abundance-based (Bray-Curtis) or presence-absence (Jaccard) metrics (**Table S3)**. Moreover, the values of the beta-diversity metrics were nearly identical regardless of binning approach (slopes ~1).

The highly correlated alpha and beta-diversity metrics indicated that results based on these metrics should yield similar ecological conclusions. Indeed, the patterns of bacterial and fungal richness and community composition across the elevation gradient were nearly indistinguishable (**Figures 2 & S1)** as were the statistical tests for both richness (**Tables S4, S5**) and community composition (**Tables S6, S7**). Moreover, family- and genus- level composition at each site along the gradient was virtually identical for bacteria **(Figure S2)** and highly similar for fungi (**Figure S3)**. We also included a mock community of eight distinct bacterial species in our PCR and sequencing runs. Both approaches resulted in highly similar mock community composition (**Figure S4)**. Thus, we found no evidence that ESVs yield better taxonomic

resolution or are more sensitive to detecting treatment effects (Callahan et al 2017). If anything, the ESV method appeared to be slightly less sensitive to detecting treatment effects on richness than the OTU method, especially for fungi in which fewer significant treatment effects were detected using ESVs **(Table S5)**.

Despite quantitative differences in microbial richness, ecological interpretation of our large bacterial and fungal community dataset was robust to the use of ESVs versus 97% OTUs. Thus, we need not question the validity of ecological results based on OTUs. Although the vagaries of molecular and bioinformatics processing inevitably add noise to microbial sequencing data, strong community-level signals will likely emerge with suitable study designs and statistics regardless of binning approach.

Conflicts of Interest: The authors declare no conflicts of interest.

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Figure 1. Comparison of observed alpha diversity for A) bacteria and B) fungi as assayed by the richness of 97% similar operational taxonomic units (OTU) versus exact sequence variants (ESV). Numbers are total observed richness after normalizing to 10,000 sequences per sample from three time points (16, 12, 18 months). Comparison of observed beta diversity for C) bacteria and D) fungi as assayed by the Bray-Curtis dissimilarity for OTUs versus ESVs from three time points (16, 12, 18 months).

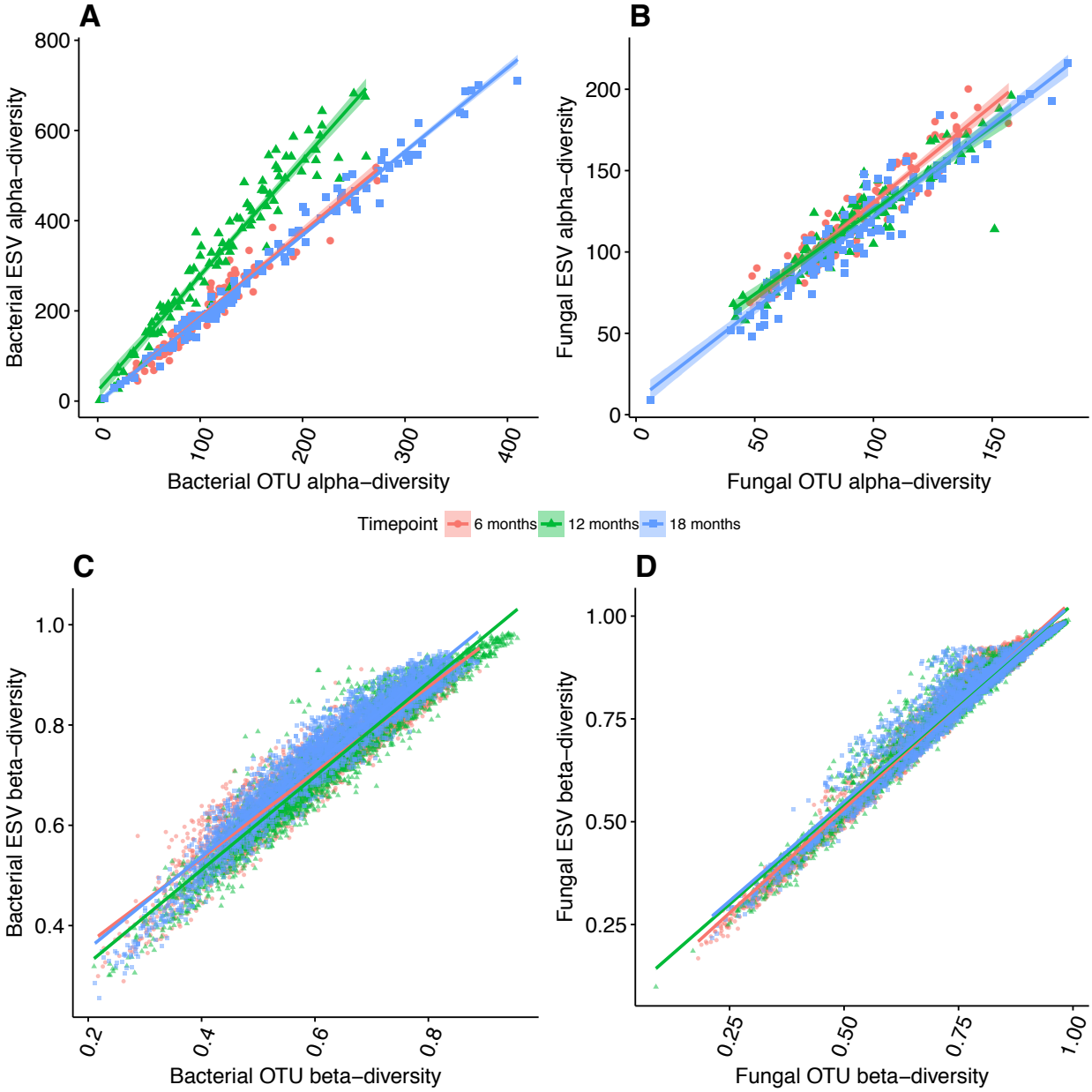
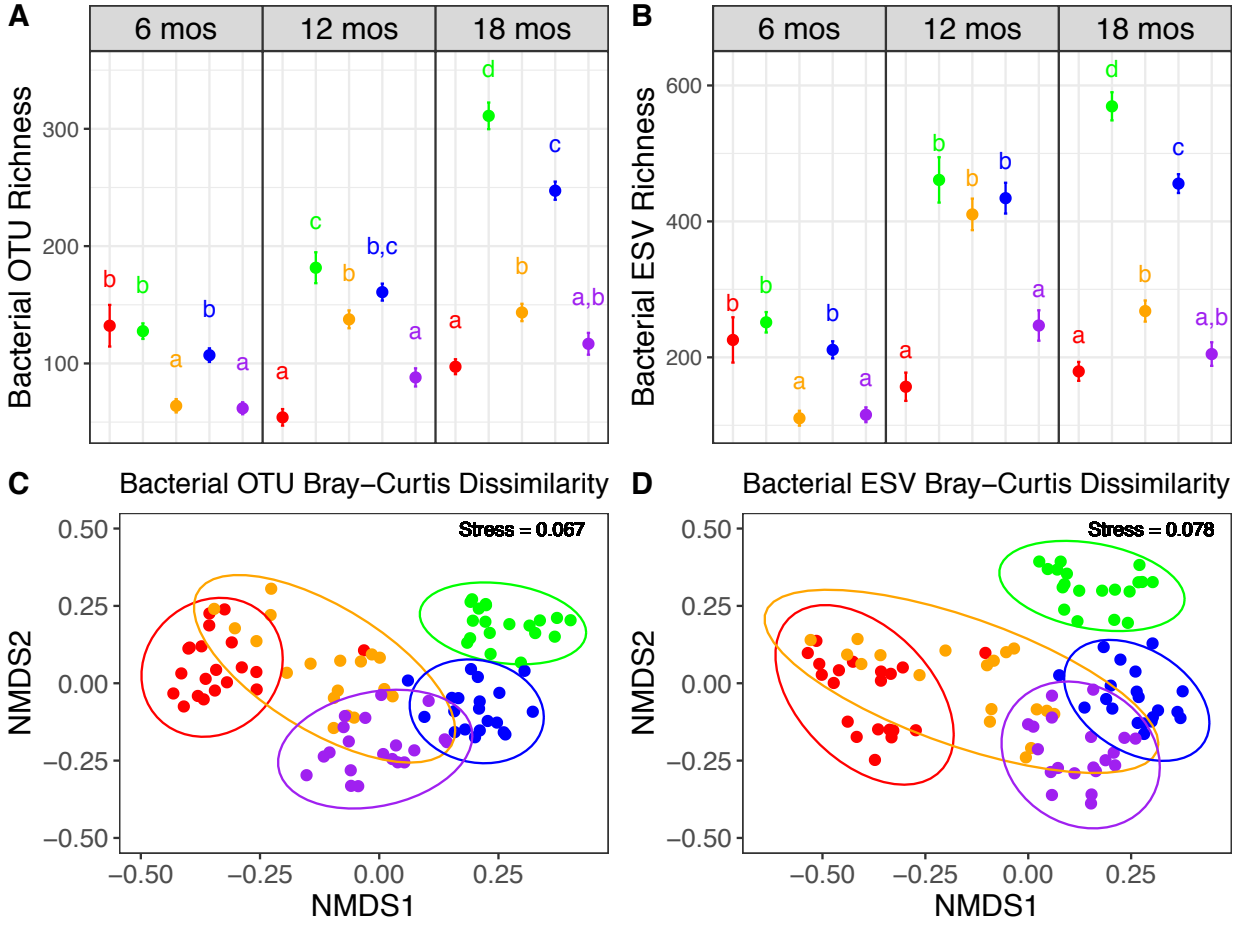


Figure 2. Comparison of alpha diversity results using A) operational taxonomic units (OTUs) versus B) exact sequencing variants (ESVs) for bacteria across elevation gradient at three time points (16, 12, 18 months). Each point represents mean observed richness per litterbag per site, and lines indicated standard error (averaged across five inoculum treatments and four replicates; n=20). Letters represent Tukey HSD significant differences across sites within a time point. Comparison of beta-diversity results using NMDS ordination of Bray-Curtis community dissimilarity of C) bacterial OTUs and D) bacterial ESVs colored by site at final time point (18 months). Ellipses represent 95% confidence intervals around the centroid. Colors represent sites along the elevation gradient ranging from lowest elevation (red; 275m) to highest elevation (purple 2240m) with middle elevation sites colored as follows: green = 470m; orange= 1280 m, blue = 1710 m).



Supplemental Information.

Title: Ecological patterns are robust to use of exact sequence variants vs operational taxonomic units

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Methods:

The dataset is derived from a reciprocal transplant experiment conducted with leaf litter across five sites ranging in elevation from 275 to 2240m in southern California, USA (Glassman et al in prep). Precipitation and temperature co-vary along this elevation gradient (275m, 470m, 1280m, 1710m, 2240m). Total precipitation throughout the duration of the experiment ranged from 213 to 1415mm and mean soil temperature ranged from 11 to 26 °C. As part of the transplant, we constructed microbial litterbags with 0.22 µm nylon mesh that allow for movement of water and nutrients but prevent dispersal of microbes (Allison et al 2013). We filled each bag with a common substrate of 5g of homogenized, gamma-irradiated, ground-up litter from a middle elevation site. We then inoculated each bag with 50mg of homogenized, ground-up litter containing the natural microbial community from each site for the initial "inoculum". At each site along the gradient, we deployed the bags into 4 replicate plots in October 2015, and collected samples at 6, 12, and 18 months until April 2017. DNA was extracted from 100 litterbags (5 sites x 5 inoculum treatments x 4 plots) at each of the three time points (n=300) using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) following the manual with the modification of adding three freeze/thaw cycles (30s in liquid Nitrogen followed by 3-5 min in 60°C water bath) prior to bead beating step to improve cell lysis.

To characterize bacterial composition, we amplified the V4 region of the 16S ribosomal RNA (rRNA) gene using the 515F-926R primers (Caporaso et al 2012) with modifications to improve diversity (Apprill et al 2015) with the forward primer as the bar-coded primer. PCR mixtures for amplification contained 0.2 µL of NEB Hot Start Taq (5 units/µL) DNA polymerase (New England Biolabs, Ipswich, MA, USA), 2.5 µL of 10× 5Prime Hotmaster buffer minus $MgCl_2$ (Quantabio, Beverly, MA, USA), 0.6 µL $MgCl_2$ (50 mM), 0.50 µL dNTPs (10mM), 0.50 µL of 10 µM non-barcoded primer, 5 µL of 1 µM barcoded primer, 0.25 µL of BSA (20mg/ml), 5 µL of DNA template (diluted to 1:10 or 1:50 to overcome inhibitors), and water up to 25 µL. PCR conditions were as follows: denaturation at 94°C for 3 min; 35 amplification cycles of 45 s at 94°C, 30 s at 55°C, 20 s at 68°C, followed by a 10-min final extension at 68°C. For the 16S libraries, we included a mock community of eight bacteria strains from Zymo Microbiomics (Zymo Research, Irvine, CA, USA) that we PCR amplified and included in each sequencing run.

To characterize fungal composition, the ITS2 region of the Internal Transcribed Spacer (ITS) was amplified using the ITS9f-ITS4 primer combination designed by (Ihrmark et al 2012) and modified for Illumina MiSeq (Looby et al 2016) following a staggered design (Tremblay et

al 2015). PCR mixtures for amplification contained 21.5 µl Platinum PCR Supermix (1.1x, Thermo Scientific, Waltham, MA, USA), 1 µl BSA (10 mg/ml, NEB, Ipswich, MA), 0.75 µl of both primers (10 µM, ITS9f and barcoded ITS4) and 1 µl of DNA template (diluted to 1:10) for a 25 µL reaction. PCR conditions were as follows: denaturation at 94°C for 5 min; 35 cycles of 45 s at 95°C, 1min at 50°C, 90 s at 72°C, followed by a 10-min final extension at 72°C.

PCR products were pooled visually according to intensity of bands based on electrophoresis gel images (scaled as weak, medium, or strong bands). Samples were pooled into six separate libraries (3 time points for each amplicon with 100-150 samples each). The pooled libraries were then purified according to the AMPure XP magnetic Bead protocol (Beckman Coulter Inc, Brea, CA, USA). For 16S, AMPure magnetic beads were used. For ITS2, we followed the same protocol but instead used a homemade solution of Sera-mag SpeedBeads (Fisher Scientific). The purified libraries were quality checked with an Agilent BioAnalyzer 2100 at the UCI Genomics High-Throughput Facility (UC Irvine, CA, USA) for size and concentration. The libraries were then sequenced in six separate Illumina MiSeq PE runs (2 x 250 bp) at the DNA Technologies Core, UC Davis Genome Center, Davis, CA, USA. Sequences were submitted to the National Center for Biotechnology Information Sequence Read Archive under accession number SRPXXXXX.

All bioinformatics processing was conducted in UPARSE (Edgar 2013) version 10 (https://www.drive5.com/usearch/manual/uparse_pipeline.html). We processed each amplicon library by each timepoint in order to examine variation in patterns among sequencing runs. We chose the UPARSE pipeline for ease of comparison of OTU versus ESV methods while keeping all other aspects of quality filtering and merging the same (Edgar 2013). First, primers were stripped, then reads were truncated based on quality, forward and reverse reads were merged, and then merged pairs were quality filtered using the fastq_filter command with a fastq_maxee parameter of 1.0. Next, at the same point in the pipeline, UPARSE can process both 97% OTUs with the "-cluster_otus" command and ESVs with the "unoise3" command. We used the default settings for each function, which for OTUs removes singletons with the "minsize 2" parameter, and the default minimum of 8 sequences per cluster for "unoise3". Both of these methods are open reference and thus capture novel diversity. OTU tables were made with the otutab command for both 97% OTUs and ESVs.

Taxonomy was assigned in QIIME 1.9 (Caporaso et al 2010) using the assign_taxonomy.py command. For 16S, assignments were made with the Greengenes database (DeSantis et al 2006) using rdp classifier and 0.80 similarity cutoff. For ITS2, we used the UNITE database (Koljalg et al 2005), accessed on June 28, 2017, using blast and minimum E value of 0.001. For ITS2, only reads mapping to kingdom Fungi were retained, and for 16S, all reads mapping to chloroplasts, mitochondria, or unclassified were removed.

Preliminary alpha diversity analyses were conducted in UPARSE. Samples were normalized to 10,000 sequences per sample using the otutab_norm command and alpha diversity metrics were calculated on the subsampled OTU tables using the alpha_div command. Next, these richness metrics were imported to R (R Core Team 2017), where we performed Pearson correlations and linear regressions to determine the correlation, intercept, and slope of the relationship between four separate alpha diversity metrics (Berger-Parker, Observed Richness, Shannon, Simpson) for both the OTU and ESV approaches for each amplicon and at each time point. We then performed ANOVA and post-hoc Tukey HSD tests to determine if the significance of our treatments on observed richness were different for OTU versus ESV for each

time point for each amplicon. All figures were made using the ggplot2 package (Wickham 2009) in the R software environment.

For beta diversity analyses, we took the raw OTU and ESV tables from UPARSE and calculated Bray-Curtis and Jaccard dissimilarity matrices for bacteria and fungi at each time point in the R package vegan (Oksanen et al 2012) using the avgdist function (https://github.com/vegandevs/vegan/blob/master/man/avgdist.Rd). Specifically, we used this function to calculate a median, square-root transformed, Bray-Curtis or Jaccard dissimilarity matrix based on 100 subsamples of either 7,000 seq/sample for bacteria or 17,000 seq/sample for fungi. We then ran Mantel correlations in vegan to test the correlation between beta-diversity metrics between the binning methods for bacteria in fungi. We visualized these correlation in ggplot. Next, PERMANOVA tests were conducted with the Adonis function in vegan to test the effects of our two treatments and their interactions on the Bray-Curtis community dissimilarity of both bacteria and fungi as assessed by either OTUs or ESVs. We then visualized these patterns with NMDS using the metaMDS function in vegan.

To determine if binning method (OTUs vs ESVs) affected distribution of taxonomic groups among each site, we summarized the top 12 most abundant families or genera at each site for the inoculum leaf litter using the dplyr package and visualized this information with barplots the ggplot2 package in R. All data and scripts to re-create all figures and statistics from paper can be found on github: https://github.com/sydneyg/OTUvESV.

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Supplemental Table:

Table S1. Summary of data on Illumina MiSeq runs. Number of reads from each of the three different runs (corresponding to the three time points 6, 12, and 18 months), number of samples, and the number of OTUs versus ESVs for fungal and bacterial amplicons.

Time Point	Amplicon	Kingdom	No. reads after quality filtering	No. Samples	No. OTUs	No. ESVs
6 months	ITS2	Fungi	5.9M	95	1196	1240
12 months	ITS2	Fungi	7.3M	95	1420	1594
18 months	ITS2	Fungi	7.3M	114	1313	1243
6 months	16S	Bacteria	3.99M	97	745	1306
12 months	16S	Bacteria	5.75M	102	1163	1999
18 months	16S	Bacteria	5.4M	119	1999	2513

Table S2. Pearson correlations and linear model relationship for 97% OTUs vs ESVs for bacteria (16S) and fungi (ITS2) for four different richness/evenness indices at each time point.

			Pearson correlation		Linear model		
Microbe	Timepoint	Metric	r	P	Slope	R^2	P
Bacteria	6 mos	Berger-Parker	0.95	<0.001	0.97	0.90	<0.001
Bacteria	12 mos	Berger-Parker	0.92	<0.001	0.97	0.84	<0.001
Bacteria	18 mos	Berger-Parker	0.95	<0.001	1.06	0.91	<0.001
Fungi	6 mos	Berger-Parker	0.94	<0.001	0.97	0.89	<0.001
Fungi	12 mos	Berger-Parker	0.92	<0.001	0.94	0.84	<0.001
Fungi	18 mos	Berger-Parker	0.97	<0.001	0.94	0.94	<0.001
Bacteria	6 mos	Richness	0.97	<0.001	0.50	0.94	<0.001
Bacteria	12 mos	Richness	0.96	<0.001	0.36	0.93	<0.001
Bacteria	18 mos	Richness	0.99	<0.001	0.53	0.99	<0.001
Fungi	6 mos	Richness	0.95	<0.001	0.75	0.90	<0.001
Fungi	12 mos	Richness	0.92	<0.001	0.82	0.85	<0.001
Fungi	18 mos	Richness	0.95	<0.001	0.80	0.91	<0.001
Bacteria	6 mos	Shannon	0.98	<0.001	0.86	0.97	<0.001
Bacteria	12 mos	Shannon	0.95	<0.001	0.77	0.90	<0.001
Bacteria	18 mos	Shannon	0.99	<0.001	0.92	0.97	<0.001
Fungi	6 mos	Shannon	0.95	<0.001	0.96	0.91	<0.001
Fungi	12 mos	Shannon	0.95	<0.001	0.90	0.89	<0.001
Fungi	18 mos	Shannon	0.98	<0.001	0.96	0.95	<0.001
Bacteria	6 mos	Simpson	0.96	<0.001	1.01	0.92	<0.001
Bacteria	12 mos	Simpson	0.93	<0.001	1.07	0.87	<0.001
Bacteria	18 mos	Simpson	0.95	<0.001	1.11	0.91	<0.001
Fungi	6 mos	Simpson	0.95	<0.001	1.07	0.90	<0.001
Fungi	12 mos	Simpson	0.93	<0.001	0.98	0.86	<0.001
Fungi	18 mos	Simpson	0.98	<0.001	1.00	0.96	<0.001

Table S3. Mantel correlations and linear model correlations for Bray-Curtis and Jaccard beta-diversity metrics calculated based on OTUs vs ESVs for each amplicon for each timepoint.

			Mantel correlation		linear model		
Microbe	Timepoint	Metric	Mantel r	P.value	Slope	R^2	P.value
Bacteria	6 mos	Bray-Curtis	0.95	0.001	0.85	0.9	<0.001
Bacteria	12 mos	Bray-Curtis	0.96	0.001	0.93	0.93	<0.001
Bacteria	18 mos	Bray-Curtis	0.96	0.001	0.93	0.93	<0.001
Fungi	6 mos	Bray-Curtis	0.99	0.001	1.02	0.98	<0.001
Fungi	12 mos	Bray-Curtis	0.98	0.001	0.98	0.96	<0.001
Fungi	18 mos	Bray-Curtis	0.97	0.001	0.97	0.95	<0.001
Bacteria	6 mos	Jaccard	0.93	0.001	0.88	0.87	<0.001
Bacteria	12 mos	Jaccard	0.94	0.001	0.93	0.88	<0.001
Bacteria	18 mos	Jaccard	0.96	0.001	0.84	0.92	<0.001
Fungi	6 mos	Jaccard	0.99	0.001	1.1	0.97	<0.001
Fungi	12 mos	Jaccard	0.98	0.001	1.04	0.97	<0.001
Fungi	18 mos	Jaccard	0.98	0.001	1.06	0.97	<0.001

Table S4. ANOVA results for bacterial OTUs vs ESVs, testing for effects of Site, Inoculum, and their interaction on richness (data plotted in Figure 2A,B).

Bacteria	**6 months**	**OTU**				
	Df	Sum Sq	Mean Sq	Fvalue	Pr(>F)	
Site	4	87281	21820	20.898	2.28E-11	***
Inoculum	4	9952	2488	2.383	5.96E-02	.
Site:Inoculum	16	71114	4445	4.257	1.11E-05	***
Residuals	70	73089	1044			

Bacteria	**6 months**	**ESV**				
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Site	4	325282	81321	20.682	2.76E-11	***
Inoculum	4	31158	7789	1.98E+00	1.07E-01	
Site:Inoculum	16	286072	17880	4.547	4.33E-06	***
Residuals	70	275243	3932			

Bacteria	**12 months**	**OTU**				
	Df	Sum Sq	Mean Sq	Fvalue	Pr(>F)	
Site	4	220757	55189	45.517	< 2e-16	***
Inoculum	4	25461	6365	5.25	0.000879	***
Site:Inoculum	16	33138	2071	1.708	0.063351	.
Residuals	75	90937	1212			

Bacteria	**12 months**	**ESV**				
	Df	Sum Sq	Mean Sq	Fvalue	Pr(>F)	
Site	4	1414191	353548	37.559	< 2e-16	***
Inoculum	4	202288	50572	5.372	0.000737	***
Site:Inoculum	16	268183	16761	1.781	0.049934	*
Residuals	75	705990	9413			

Bacteria	**18 months**	**OTU**				
	Df	Sum Sq	Mean Sq		Pr(>F)	
Site	4	660280	165070	134.294	< 2e-16	***
Inoculum	4	20634	5159	4.197	0.00407	**
Site:Inoculum	16	24418	1526	1.242	2.59E-01	
Residuals	74	90958	1229			

Bacteria	**18 months**	**ESV**				
	Df	Sum Sq	Mean Sq	Fvalue	Pr(>F)	
Site	4	2246528	561632	118.012	<2e-16	***

Inoculum	4	53361	13340	2.803	0.0317	*
Site:Inoculum	16	93870	5867	1.233	2.65E-01	
Residuals	74	352173	4759			

Table S5. ANOVA results for fungal OTUs vs ESVs, testing for effects of Site, Inoculum, and their interaction on richness (data plotted in Figure S1A,B).

Fungi	**6 months**	**OTU**				
	Df	Sum Sq	Mean Sq	Fvalue	Pr(>F)	
Site	4	6163	1540.9	4.265	0.00377	**
Inoculum	4	12633	3158.3	8.741	8.53E-06	***
Site:Inoculum	16	13216	826	2.286	0.00926	**
Residuals	71	25654	361.3			

Fungi	**6 months**	**ESV**				
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Site	4	3936	984	1.763	0.14594	
Inoculum	4	24459	6115	1.10E+01	5.66E-07	***
Site:Inoculum	16	23398	1462	2.62	0.00289	**
Residuals	71	39636	558			

Fungi	**12 months**	**OTU**				
	Df	Sum Sq	Mean Sq	Fvalue	Pr(>F)	
Site	4	12921	3230	7.075	7.64E-05	***
Inoculum	4	7172	1793	3.927	0.00621	**
Site:Inoculum	16	9612	601	1.316	0.21263	
Residuals	70	31959	457			

Fungi	**12 months**	**ESV**				
	Df	Sum Sq	Mean Sq	Fvalue	Pr(>F)	
Site	4	10530	2632.6	3.959	0.00593	**
Inoculum	4	6069	1517.3	2.282	0.06907	.
Site:Inoculum	16	13794	862.1	1.296	0.22435	
Residuals	70	46550	665			

Fungi	**18 months**	**OTU**				
	Df	Sum Sq	Mean Sq		Pr(>F)	
Site	4	13800	3450	8.898	7.22E-06	***
Inoculum	4	9753	2438	6.289	0.000221	***
Site:Inoculum	16	27154	1697	4.377	7.50E-06	***
Residuals	70	27141	388			

Fungi	**18 months**	**ESV**				
	Df	Sum Sq	Mean Sq	Fvalue	Pr(>F)	
Site	4	15399	3850	6.472	0.000172	***

Inoculum	4	10599	2650	4.455	0.002894	**
Site:Inoculum	16	36416	2276	3.826	4.63E-05	***
Residuals	70	41636	595			

Table S6. PERMANOVA results for Bacterial OTUs vs ESVs, testing for effects of Site, Inoculum, and their interaction on Bray-Curtis community dissimilarity (data plotted in Figure 2C,D).

6 months	OTU						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Site	4	5.3289	1.33222	17.4236	0.34467	1.00E-04	***
Inoculum	4	2.5453	0.63633	8.3224	0.16463	1.00E-04	***
Site:Inoculum	16	2.9225	0.18266	2.3889	0.18903	1.00E-04	***
Residuals	61	4.6641	0.07646		0.30167		
Total	85	15.4608			1		

6 months	ESV						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Site	4	5.7313	1.43282	12.4584	0.27056	1.00E-04	***
Inoculum	4	4.0616	1.01539	8.8288	0.19174	1.00E-04	***
Site:Inoculum	16	4.3744	0.2734	2.3772	0.20651	1.00E-04	***
Residuals	61	7.0155	0.11501		0.33119		
Total	85	21.1828			1		

12 months	OTU						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Site	4	6.8983	1.72459	19.7618	0.35664	1.00E-04	***
Inoculum	4	2.9448	0.7362	8.436	0.15224	1.00E-04	***
Site:Inoculum	16	3.216	0.201	2.3032	0.16627	1.00E-04	***
Residuals	72	6.2834	0.08727		0.32485		
Total	96	19.3425			1		

12 months	ESV						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Site	4	8.0058	2.00144	15.9659	0.31394	1.00E-04	***
Inoculum	4	3.9817	0.99542	7.9406	0.15614	1.00E-04	***
Site:Inoculum	16	4.4875	0.28047	2.2374	0.17598	1.00E-04	***
Residuals	72	9.0257	0.12536		0.35394		
Total	96	25.5007			1		

18 months	OTU						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Site	4	8.8405	2.21013	28.6693	0.4675	1.00E-04	***
Inoculum	4	1.9093	0.47732	6.1916	0.10096	1.00E-04	***

Site:Inoculum	16	2.6101	0.16313	2.1161	0.13802	1.00E-04	***
Residuals	72	5.5505	0.07709		0.29352		
Total	96	18.9104			1		

18 months ESV

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Site	4	10.228	2.55701	21.3118	0.38457	1.00E-04	***
Inoculum	4	3.3904	0.84761	7.0645	0.12748	1.00E-04	***
Site:Inoculum	16	4.3388	0.27117	2.2601	0.16314	1.00E-04	***
Residuals	72	8.6386	0.11998		0.32481		
Total	96	26.5958			1		

Table S7. PERMANOVA results for fungal OTUs vs ESVs, testing for effects of Site, Inoculum, and their interaction on Bray-Curtis community dissimilarity (data plotted in Figure S1C,D).

6 months	OTU						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Site	4	3.6505	0.9126	16.292	0.13819	1.00E-04	***
Inoculum	4	15.1216	3.7804	67.486	0.57242	1.00E-04	***
Site:Inoculum	16	3.8354	0.2397	4.279	0.14519	1.00E-04	***
Residuals	68	3.8092	0.056		0.1442		
Total	92	26.4167			1		

6 months	ESV						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Site	4	3.4677	0.8669	14.285	0.11983	1.00E-04	***
Inoculum	4	17.1383	4.2846	70.599	0.59222	1.00E-04	***
Site:Inoculum	16	4.2065	0.2629	4.332	0.14536	1.00E-04	***
Residuals	68	4.1268	0.0607		0.1426		
Total	92	28.9393			1		

12 months	OTU						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Site	4	3.8139	0.95347	10.747	0.15252	1.00E-04	***
Inoculum	4	11.3907	2.84767	32.096	0.45552	1.00E-04	***
Site:Inoculum	16	4.123	0.25769	2.904	0.16488	1.00E-04	***
Residuals	64	5.6782	0.08872		0.22708		
Total	88	25.0058			1		

12 months	ESV						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Site	4	3.6894	0.9223	9.676	0.13472	1.00E-04	***
Inoculum	4	12.9589	3.2397	33.987	0.47322	1.00E-04	***
Site:Inoculum	16	4.6356	0.2897	3.039	0.16928	1.00E-04	***
Residuals	64	6.1006	0.0953		0.22278		
Total	88	27.3845			1		

18 months	OTU						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Site	4	5.111	1.27775	15.693	0.20105	1.00E-04	***
Inoculum	4	10.498	2.62451	32.234	0.41296	1.00E-04	***
Site:Inoculum	16	4.5197	0.28248	3.469	0.17779	1.00E-04	***

Residuals	65	5.2924	0.08142		0.20819		
Total	89	25.4212			1		

18 months ESV

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Site	4	4.7856	1.19641	13.118	0.17019	1.00E-04	***
Inoculum	4	12.1426	3.03564	33.284	0.43183	1.00E-04	***
Site:Inoculum	16	5.2625	0.32891	3.606	0.18715	1.00E-04	***
Residuals	65	5.9283	0.0912		0.21083		
Total	89	28.119			1		

Supplemental Figures S1-S4

Figure S1. Comparison of alpha diversity results using A) operational taxonomic units (OTUs) versus B) exact sequencing variants (ESVs) for fungi across elevation gradient at three time points (16, 12, 18 months). Each point represents mean richness per litterbag per site (averaged across five inoculum treatments and four replicates; n=20). Letters represent Tukey HSD significant differences across sites within a time point. Comparison of beta-diversity results using NMDS ordination of Bray-Curtis community dissimilarity of A) fungal OTUs and b) fungal ESVs colored by inoculum at final time point (18 months). Ellipses represent 95% confidence intervals around the centroid. Colors represent inoculum from sites along the elevation gradient ranging from lowest elevation (red; 275m) to highest elevation (purple 2250m).

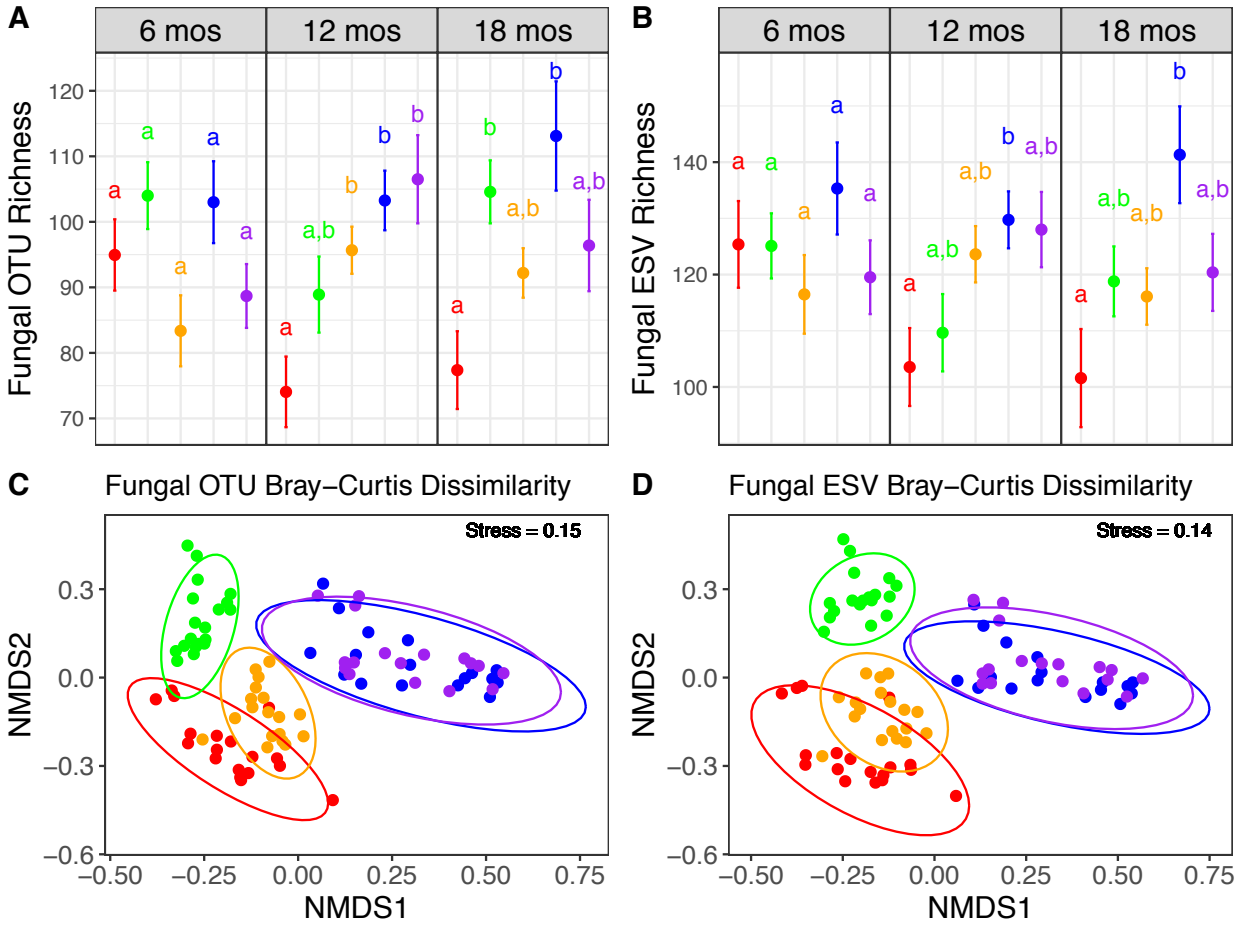
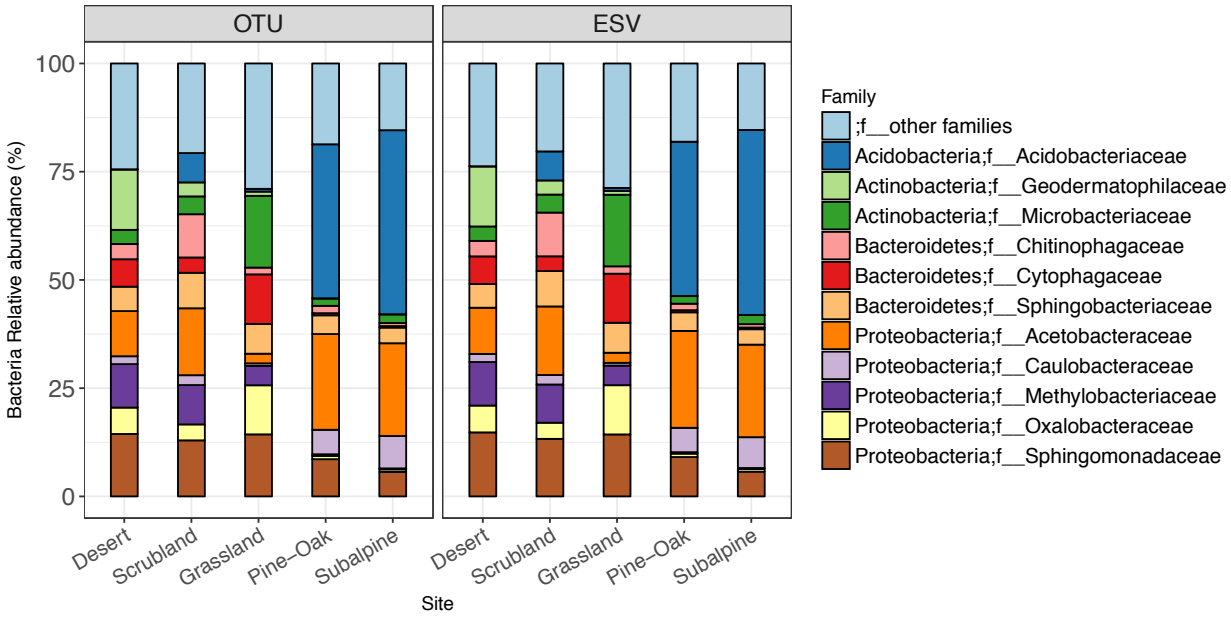


Figure S2. Relative abundance of bacterial sequences per sample of the inoculum leaf litter from each site (n=20) for OTUs versus ESVs summarized by A) the 12 most abundant families or B) the 12 most abundant genera.

A)



B)

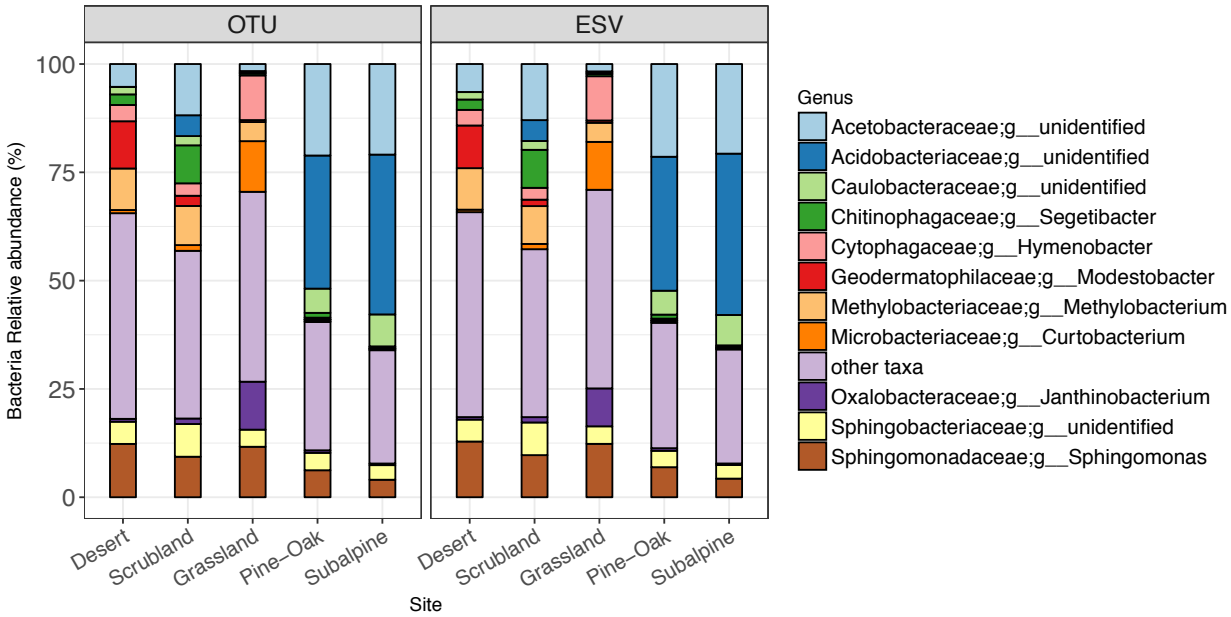
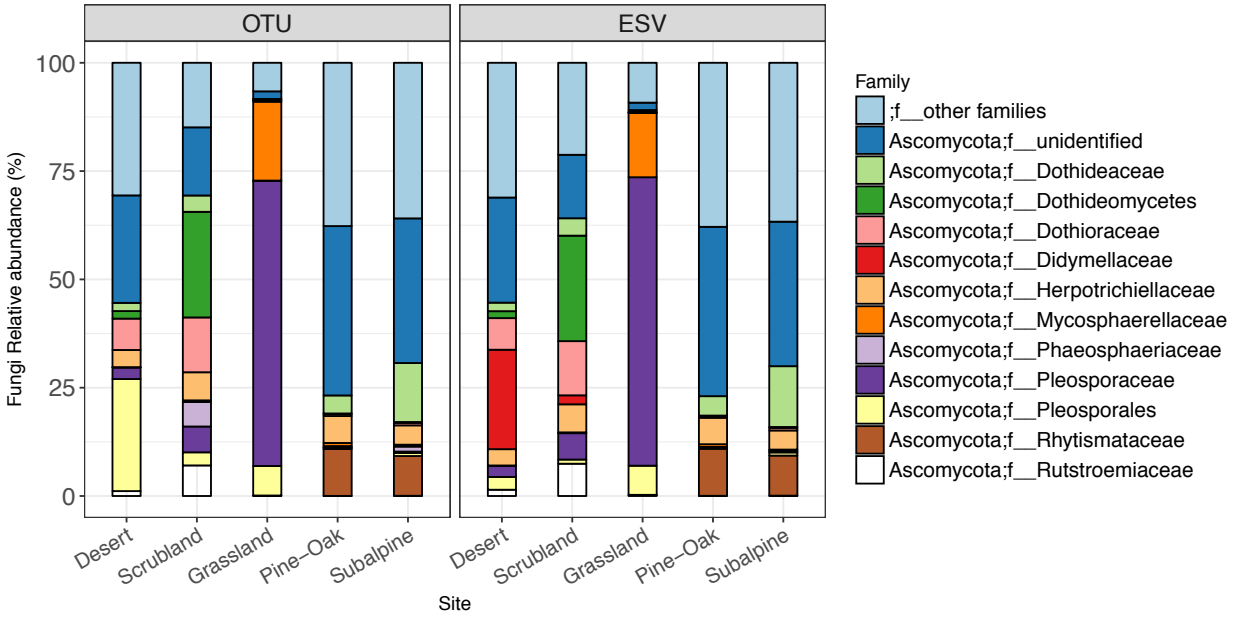


Figure S3. Relative abundance of fungal sequences per sample of the inoculum leaf litter from each site (n=20) for OTUs versus ESVs summarized by A) the 12 most abundant families (or next best taxonomic classification) or B) the 12 most abundant genera.

A)



B)

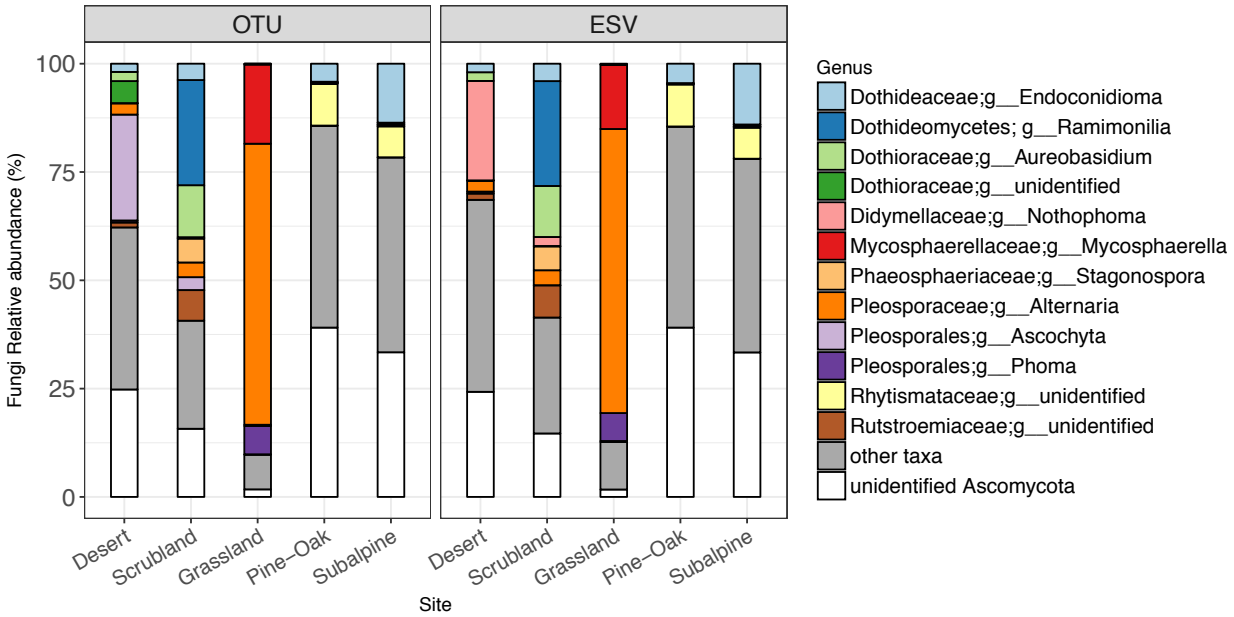
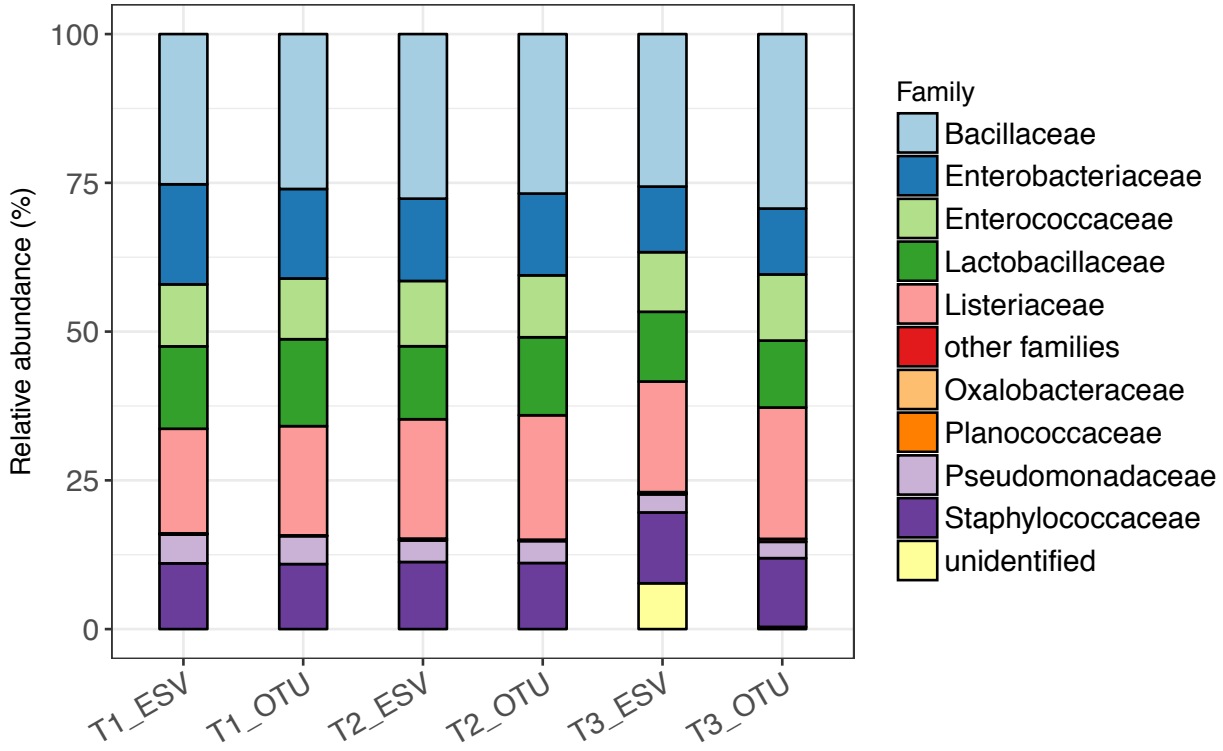



Figure S4. Relative abundance of bacterial sequences per mock community sample from each Illumina MiSeq run for each time point with either ESVs or 97% OTUs. The three timepoints represent 6 months (T1), 12 months (T2) and 18 months (T3) after transplantation. Both ESVs and OTUs largely recapitulated the eight mock community taxa at the family level, although in both cases more than 8 taxa were found. Bacterial taxa included in th mock community were: *Bacillus subtilis (F. Bacillaceae), Escherichia coli (F. Enterobacteriaceae), Salmonella enterica (F. Enterobacteriaceae), Enterococcus faecalis (F. Enterococcaceae), Lactobacillus fermentum (F. Lactobacillaceae), Pseudomonas aeruginosa (F. Pseudomonadaceae;), Staphylococcus aureus (F. Staphylococcaceae), Listeria monocytogenes (F. Listeriaceae).* For ESVs, there were more taxa within each of these dominant families than for OTUs, but since all the taxa included in the mock community have 16S multiple copies, it is unclear if these are truly spurious taxa or real genetic variation within the 16S gene (for information on taxa in mock community see: https://d2gsy6rsbfrvyb.cloudfront.net/media/amasty/amfile/attach/_D6300_ZymoBIOMICS_Microbial_Community_Standard_v1.1.3.pdf). For both OTUs and ESVs, there were low abundance sequences from families common in the experimental samples (i.e. Oxalobacteraceae), and these likely represent spillover between the barcoded samples.