Title: Broad-scale alpha- and beta- diversity 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 defined by ≥97% sequence similarity) affected the conclusions of a large field study. The dataset included sequences targeting all 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. Consequently, statistical inferences were nearly indistinguishable. Further, ESVs only moderately increased the genetic resolution of fungal and bacterial diversity (1.3 and 2.1 times OTU richness, respectively). We conclude that for broad-scale (e.g., all bacteria or all fungi) alpha- and beta-diversity analyses, OTU or ESV methods will often reveal similar ecological results. Thus, while there are good reasons to employ ESVs, we need not question the validity of results based on OTUs. There are advantages and disadvantages of both, and which binning method to employ will depend on question/data in hand.

Importance: Microbial ecologists have made exceptional improvements in our understanding of microbiomes in the last decade due to breakthroughs in sequencing technologies. These advances

have wide ranging implications for fields ranging from agriculture to human health. Due to limitations in databases, the majority of microbial ecology studies use a binning approach to approximate taxonomy based on DNA sequence similarity. There remains extensive debate on the best way to bin and approximate this taxonomy. Here we examine two popular approaches using a large field based data set examining both bacteria and fungi, and conclude that there are not major differences in the ecological conclusions. Thus, despite controversy, it appears that standard microbial community analyses may not be very sensitive to the particulars of binning approaches.

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 (1) to sequencing platform (2) are contested. Further downstream, the options for computational processing of amplicon sequences are similarly deliberated (e.g. (3-5)). Yet despite these ongoing debates, microbial ecology has made great strides towards characterizing and testing hypotheses in environmental and host-associated microbiomes (e.g. (6, 7)).

Within microbiome studies, operational taxonomic units (OTUs) have been used to delineate microbial taxa, as microbial diversity still vastly outstrips our global databases (8). 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 (9) and a recognition of spurious diversity accumulated through PCR and sequencing errors (10, 11).

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)(12) or zOTUs (13), 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 (13-15). Moreover, proponents have stated that ESVs should replace OTUs altogether (12), and some journal reviewers are insisting that ESVs be used over OTUs as a condition for a study's publication. However, as with the adoption of any new approach, there remains a need to quantify how the ESV method compares to the large body of previous research based on 97% OTUs. Further, OTU classifications remain biologically useful for comparing diversity across large datasets (7) or identifying clades that share traits (16).

Here, we tested if use of ESVs versus 97% OTUs affected the ecological conclusions, including treatment effects and alpha- and beta- diversity patterns, and if these conclusions varied based on the amplicon targeted. 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 (17). We sequenced both bacteria (16S) and fungi (ITS2) from litterbags collected at three time points (6, 12, and 18 months after deployment) in separate Illumina MiSeq 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, but that results might differ based on amplicon sequenced.

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

76 approaches by keeping all other aspects of quality filtering and merging consistent (4). ESV and
77 OTU alpha-diversity was strongly correlated across samples using four metrics for both bacteria
78 and fungi (mean Pearson r = 0.95 ± 0.02; all P values < 0.001). For the three metrics (Berger-
79 Parker, Shannon, Simpson), the ESV and OTU approaches were not only highly correlated
80 (mean Pearson r = 0.95 ± 0.02), but nearly equivalent in their values (mean slope = 0.97; **Table**
81 **S2**). For observed richness, ESV versus OTU was also highly correlated across all time
82 points/sequencing runs (Pearson r > 0.92; **Figure 1A,B**). However, bacterial OTU richness was
83 approximately half that of ESV richness for the same sample (mean slope = 0.46), and fungal
84 OTU richness was approximately three quarters that of ESV richness (mean slope = 0.79). We
85 speculate that this difference between bacteria and fungi is due to the coarser phylogenetic
86 breadth of the 16S versus ITS genetic regions.

87 Beta-diversity metrics were also strongly correlated across samples for ESVs and OTUs
88 (Bray-Curtis average Mantel r = 0.96 for bacteria and 0.98 for fungi; all P < 0.01; **Figure 1C,D**),
89 whether assessed by abundance-based (Bray-Curtis) or presence-absence (Jaccard) metrics
90 (**Table S2)**. Moreover, the values of the beta-diversity metrics were nearly identical regardless of
91 binning approach (slopes ~1).

92 The highly correlated alpha and beta-diversity metrics indicated that results based on
93 these metrics should yield similar ecological conclusions. Indeed, the patterns of bacterial and
94 fungal richness and community composition across the elevation gradient were nearly
95 indistinguishable (**Figures 2 & S1)** as were the statistical tests for both richness (**Tables S3**) and
96 community composition (**Tables S4, S5**). Moreover, family- and genus- level composition at
97 each site along the gradient was virtually identical for bacteria **(Figure S2)** and highly similar for
98 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 (12). 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 S3)**.

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, even though there are good reasons to take an ESV approach, we need not question the validity of studies based on 97% OTUs. Indeed, while previous studies have found that ESVs can help explain additional variation among samples (18, 19), the general alpha- and beta-diversity patterns of ESVs and OTUs in these studies were also quite similar. In general, we suspect that the robustness of such comparisons will vary depending on the breadth of the microbial community targeted. For instance, here we characterized all bacteria and fungi in a diverse environmental community, as opposed to a narrower subset of taxa or a less diverse host-associated community.

It is clear that both 97% OTUs and ESVs mask ecologically important trait variation of individual taxa (18, 20). In our study, ESVs only slightly increased the detection of fungal and bacterial diversity (1.3 and 2.1 times OTU richness, respectively), highlighting that ribosomal marker genes at any resolution are generally poor targets for improving genetic resolution within a microbial community. For example, it is widely known that many taxa can share the same 16S (20) or ITS (21). Thus, if strain identification is critical, then a full genome (22) or amplicon of a less conservative marker gene (23) is required. But for broad-scale community alpha and beta-

diversity patterns, 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 captions.

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).

240 **Figure 1.**

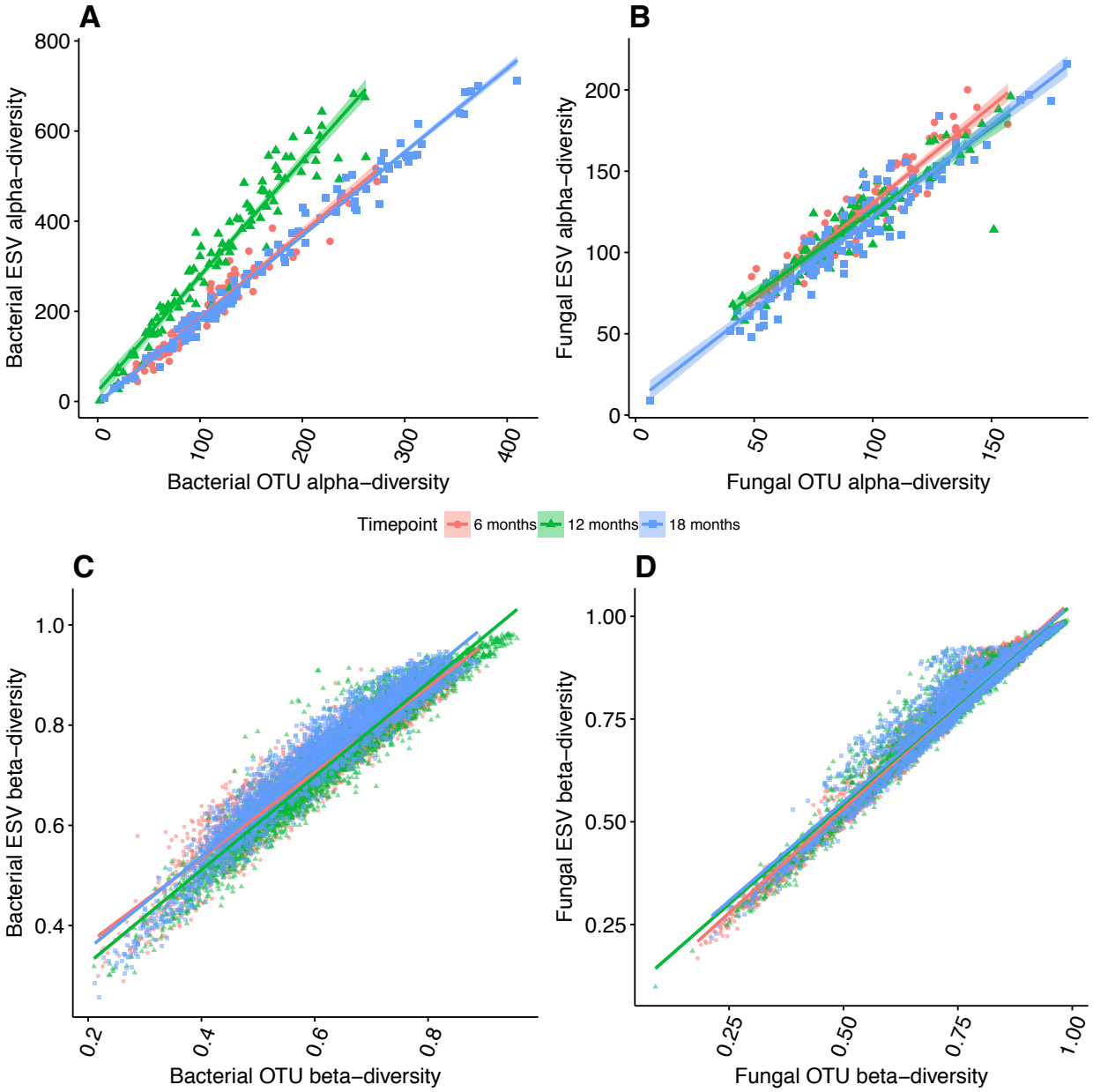

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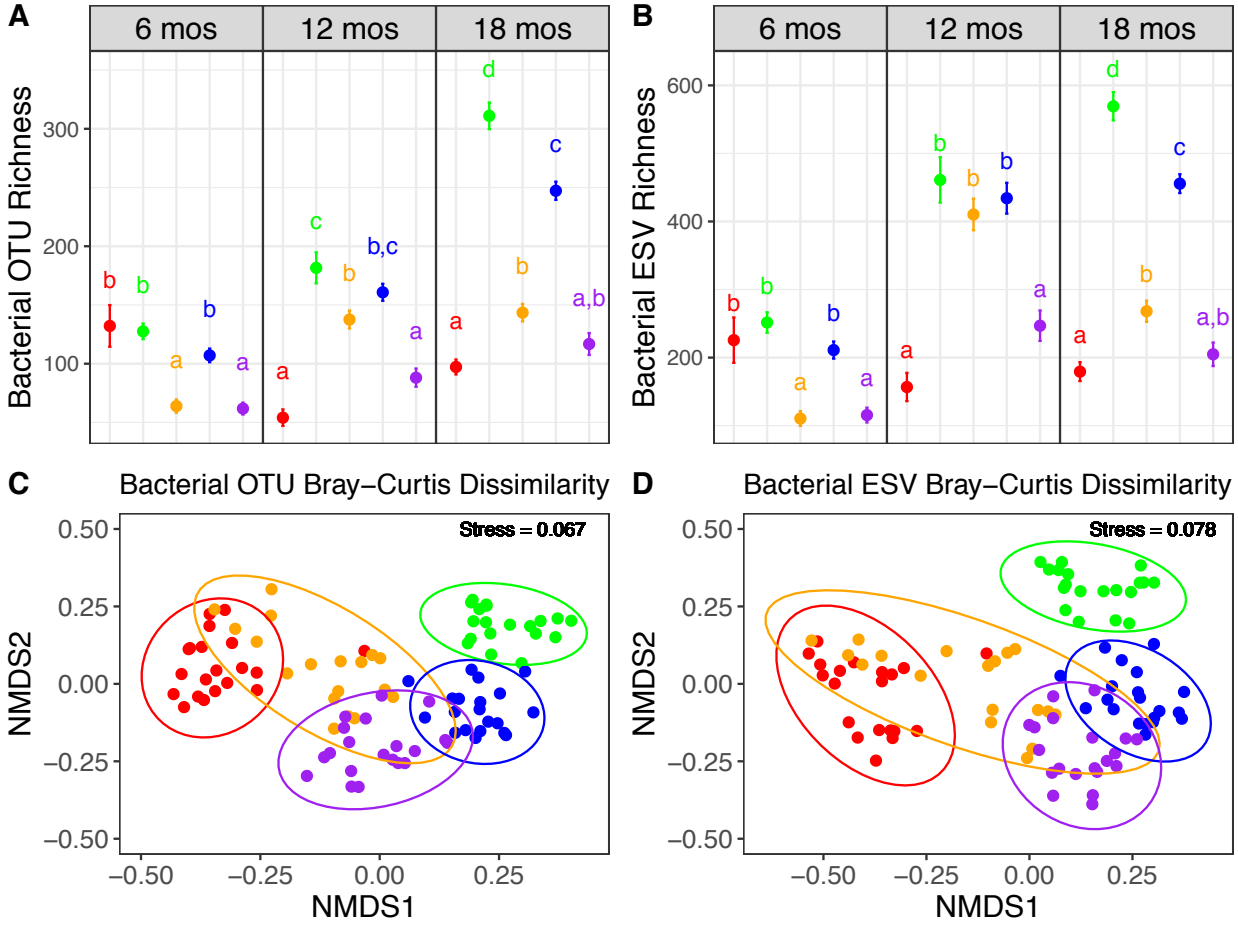
Figure 2.


A
6 mos
12 mos
18 mos
Bacterial OTU Richness
B
Bacterial ESV Richness
C
Bacterial OTU Bray–Curtis Dissimilarity
Stress = 0.067
NMDS2
NMDS1
D
Bacterial ESV Bray–Curtis Dissimilarity
Stress = 0.078

Supplemental Information.

Title: Broad-scale alpha- and beta- diversity patterns are robust to use of exact sequence variants versus 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 (1). 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 (2) with modifications to improve diversity (3) 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 (4) and modified for Illumina MiSeq (5) following a staggered design (6). 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

304 of DNA template (diluted to 1:10) for a 25 µL reaction. PCR conditions were as follows:
305 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
306 by a 10-min final extension at 72°C.
307 PCR products were pooled visually according to intensity of bands based on
308 electrophoresis gel images (scaled as weak, medium, or strong bands). Samples were pooled into
309 six separate libraries (3 time points for each amplicon with 100-150 samples each). The pooled
310 libraries were then purified according to the AMPure XP magnetic Bead protocol (Beckman
311 Coulter Inc, Brea, CA, USA). For 16S, AMPure magnetic beads were used. For ITS2, we
312 followed the same protocol but instead used a homemade solution of Sera-mag SpeedBeads
313 (Fisher Scientific). The purified libraries were quality checked with an Agilent BioAnalyzer
314 2100 at the UCI Genomics High-Throughput Facility (UC Irvine, CA, USA) for size and
315 concentration. The libraries were then sequenced in six separate Illumina MiSeq PE runs (2 x
316 250 bp) at the DNA Technologies Core, UC Davis Genome Center, Davis, CA, USA. Sequences
317 were submitted to the National Center for Biotechnology Information Sequence Read Archive
318 under accession number SRPXXXXX.
319 All bioinformatics processing was conducted in UPARSE (7) version 10
320 (https://www.drive5.com/usearch/manual/uparse_pipeline.html). We processed each amplicon
321 library by each timepoint in order to examine variation in patterns among sequencing runs. We
322 chose the UPARSE pipeline for ease of comparison of OTU versus ESV methods while keeping
323 all other aspects of quality filtering and merging the same (7). First, primers were stripped, then
324 reads were truncated based on quality, forward and reverse reads were merged, and then merged
325 pairs were quality filtered using the fastq_filter command with a fastq_maxee parameter of 1.0.
326 Next, at the same point in the pipeline, UPARSE can process both 97% OTUs with the "-
327 cluster_otus" command and ESVs with the "unoise3" command. We used the default settings for
328 each function, which for OTUs removes singletons with the "minsize 2" parameter, and the
329 default minimum of 8 sequences per cluster for "unoise3". Both of these methods are open
330 reference and thus capture novel diversity. OTU tables were made with the otutab command for
331 both 97% OTUs and ESVs.
332 Taxonomy was assigned in QIIME 1.9 (8) using the assign_taxonomy.py command. For
333 16S, assignments were made with the Greengenes database (9) using rdp classifier and 0.80
334 similarity cutoff. For ITS2, we used the UNITE database (10), accessed on June 28, 2017, using
335 blast and minimum E value of 0.001. For ITS2, only reads mapping to kingdom Fungi were
336 retained, and for 16S, all reads mapping to chloroplasts, mitochondria, or unclassified were
337 removed.
338 Preliminary alpha diversity analyses were conducted in UPARSE. Samples were
339 normalized to 10,000 sequences per sample using the otutab_norm command and alpha diversity
340 metrics were calculated on the subsampled OTU tables using the alpha_div command. Next,
341 these richness metrics were imported to R (11), where we performed Pearson correlations and
342 linear regressions to determine the correlation, intercept, and slope of the relationship between
343 four separate alpha diversity metrics (Berger-Parker, Observed Richness, Shannon, Simpson) for
344 both the OTU and ESV approaches for each amplicon and at each time point. We then performed
345 ANOVA and post-hoc Tukey HSD tests to determine if the significance of our treatments on
346 observed richness were different for OTU versus ESV for each time point for each amplicon. All
347 figures were made using the ggplot2 package (12) in the R software environment.
348 For beta diversity analyses, we took the raw OTU and ESV tables from UPARSE and
349 calculated Bray-Curtis and Jaccard dissimilarity matrices for bacteria and fungi at each time

point in the R package vegan (13) 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 Information 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).

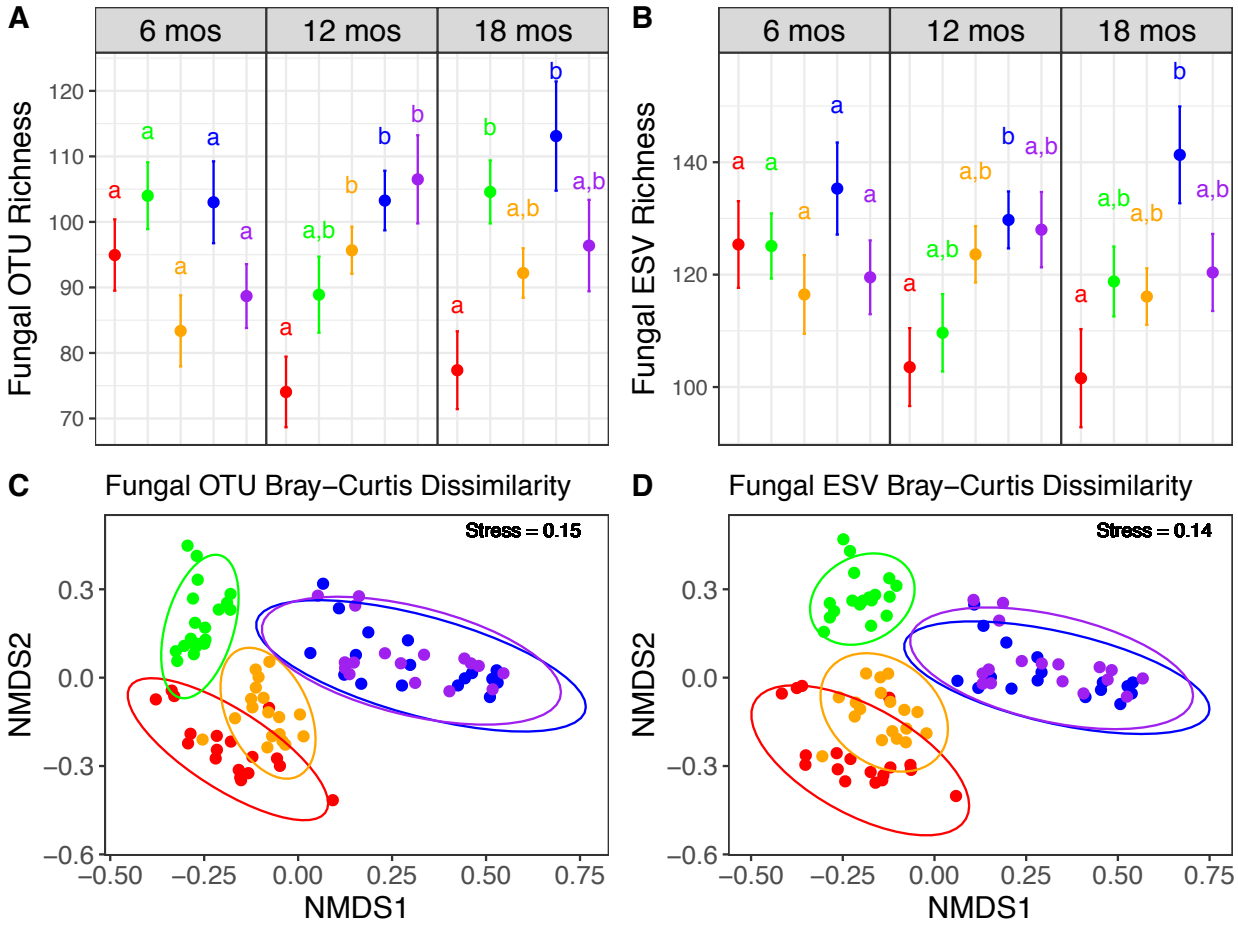
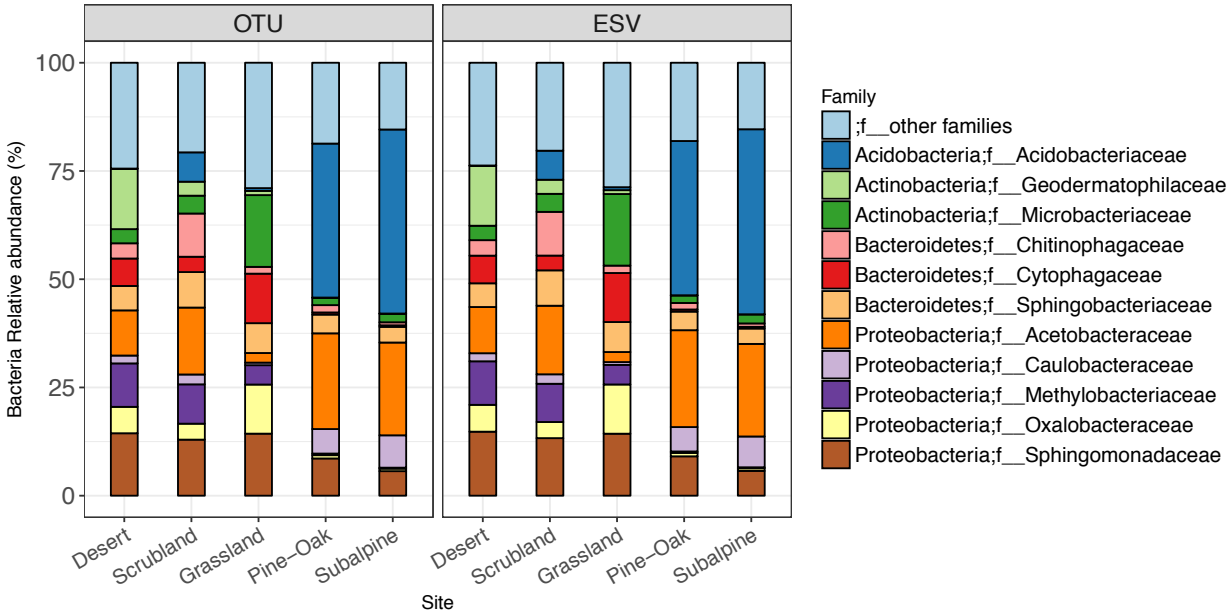


Figure S2. Relative abundance of bacterial sequences per sample of the inoculum leaf litter from each site (n=20) for OTUs versus ESVs summarized by A) the 12 most abundant families or B) the 12 most abundant genera.

A)



B)

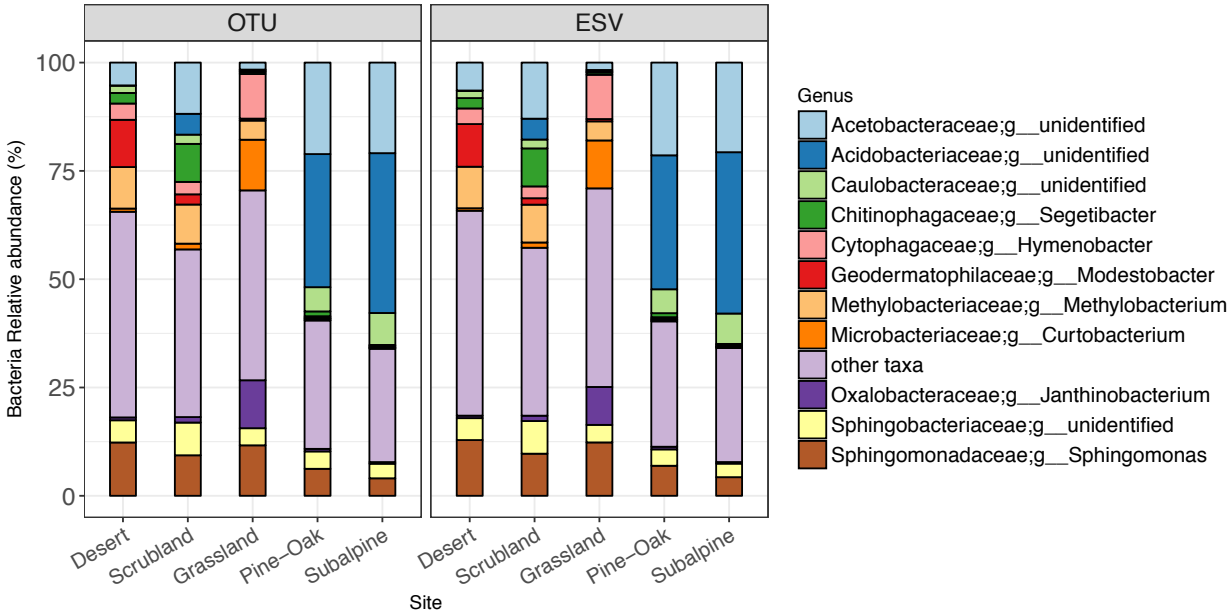
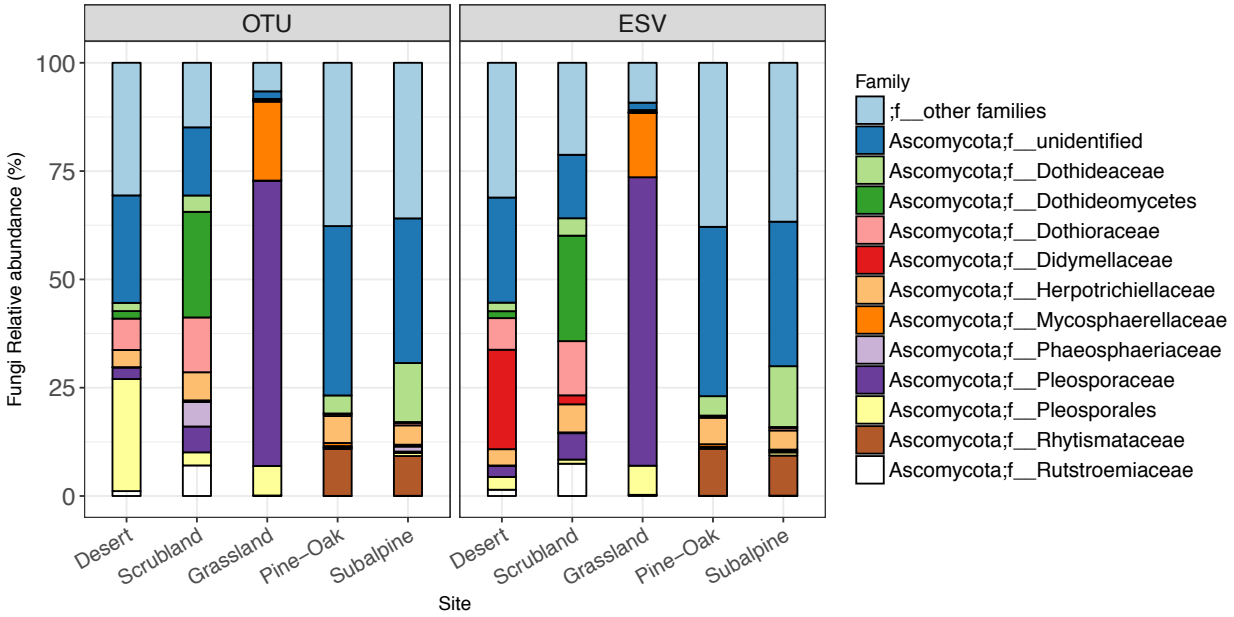


Figure S3. Relative abundance of fungal sequences per sample of the inoculum leaf litter from each site (n=20) for OTUs versus ESVs summarized by A) the 12 most abundant families (or next best taxonomic classification) or B) the 12 most abundant genera.

A)



B)

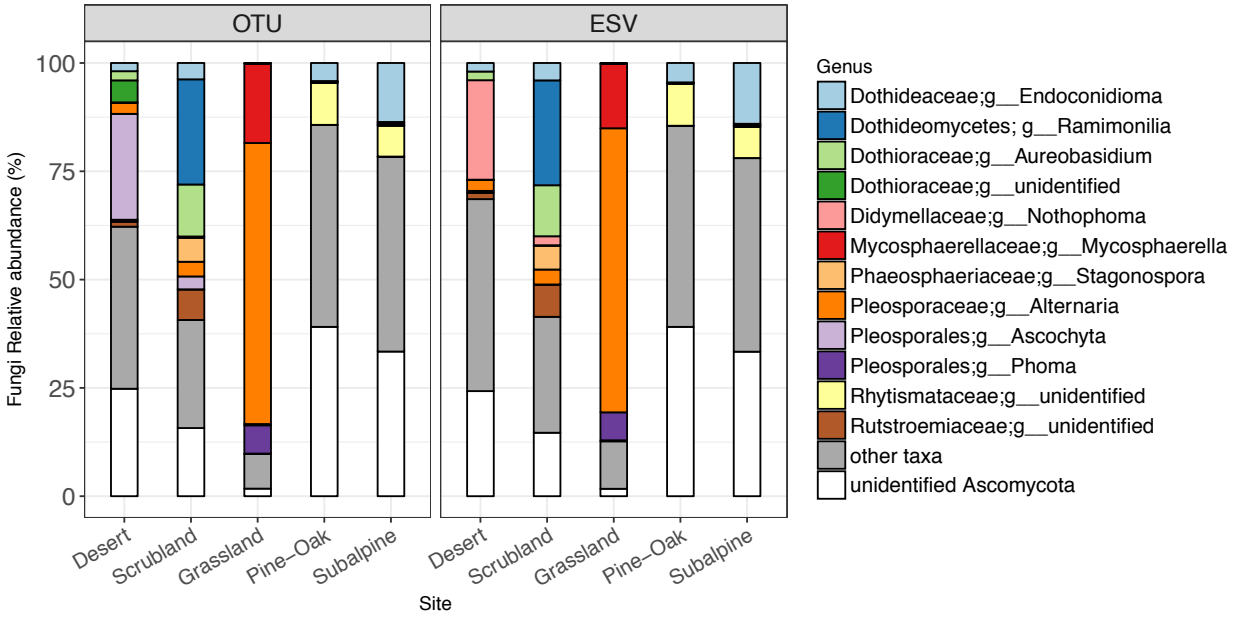
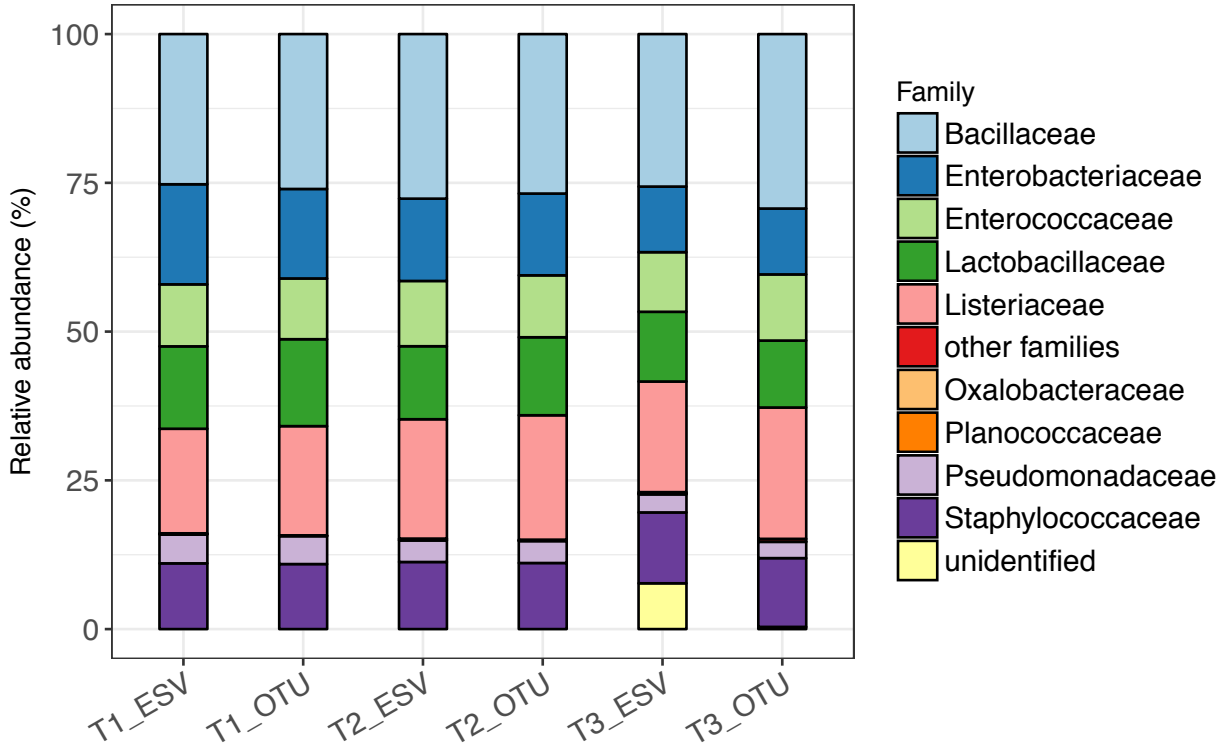


Figure S4. Relative abundance of bacterial sequences per mock community sample from each Illumina MiSeq run for each time point with either ESVs or 97% OTUs. The three timepoints represent 6 months (T1), 12 months (T2) and 18 months (T3) after transplantation. Both ESVs and OTUs largely recapitulated 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.



Supplemental Information Tables S1-S5

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 relationships for 97% OTUs vs ESVs for alpha-diversity and beta-diversity metrics for bacteria (16S) and fungi (ITS2) by time point.

Alpha-Diversity			**Pearson correlation**		**Linear model**		
Microbe	Timepoint	Metric	r	P	Slope	R^2	P
Bacteria	6 mos	Berger-Parker	0.95	<0.001	0.97	0.9	<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	0.5	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	0.9	<0.001
Fungi	12 mos	Richness	0.92	<0.001	0.82	0.85	<0.001
Fungi	18 mos	Richness	0.95	<0.001	0.8	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	0.9	<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	0.9	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	0.9	<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	0.96	<0.001
Beta-Diversity			**Mantel correlation**		**Linear model**		
Microbe	Timepoint	Metric	Mantel r	P.value	Slope	R^2	P
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 S3. ANOVA results for OTUs and ESVs at each of the three time points, testing for effects of Site, Inoculum, and their interaction on bacterial or fungal richness.

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			

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 S4. 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 S5. 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		