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ARTICLE

# Microbes on decomposing litter in streams: entering on the leaf or colonizing in the water?

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When leaves fall in rivers, microbial decomposition commences within hours. Microbial assemblages comprising hundreds of species of fungi and bacteria can vary with stream conditions, leaf litter species, and decomposition stage. In terrestrial ecosystems, fungi and bacteria that enter soils with dead leaves often play prominent roles in decomposition, but their role in aquatic decomposition is less known. Here, we test whether fungi and bacteria that enter streams on senesced leaves are growing during decomposition and compare their abundances and growth to bacteria and fungi that colonize leaves in the water. We employ quantitative stable isotope probing to identify growing microbes across four leaf litter species and two decomposition times. We find that most of the growing fungal species on decomposing leaves enter the water with the leaf, whereas most growing bacteria colonize from the water column. Results indicate that the majority of bacteria found on litter are growing, whereas the majority of fungi are dormant. Both bacterial and fungal assemblages differed with leaf type on the dried leaves and throughout decomposition. This research demonstrates the importance of fungal species that enter with the leaf on aquatic decomposition and the prominence of bacteria that colonize decomposing leaves in the water.

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## INTRODUCTION

Leaf litter inputs into terrestrial and aquatic habitats drive biogeochemical cycles and support large food webs. Microbial assemblages, comprising hundreds of fungal and bacterial taxa, play a prominent role in litter decomposition in most ecosystems. Molecular tools have revolutionized our understanding of microbes associated with leaf litter, revealing far greater species diversity, phylogenetic breadth and life history complexity than morphological studies. In terrestrial ecosystems, molecular tools have revealed that many fungi found on decomposing litter also live in the phyllosphere, the assemblage of endophytes and epiphytes on living or recently senesced leaves [1, 2]. The importance of the phyllosphere in decomposition has been documented in multiple terrestrial ecosystems where species can alternate between being biotrophic and saprotrophic [3–7]. A facultative trophic strategy where species capitalize on the last opportunity to obtain carbon from a living host and the first to consume its tissues upon its death confers a priority effect during decomposition [8], an effect that can also shape the composition of fungi that colonize decomposing leaves from the soil [7, 9]. The relative abundance of fungal species on decomposing litter entering with the phyllosphere sometimes decreases as decomposition progresses, with obligate saprotrophs dominating later successional stages [4, 5], but the imprint of the initial assemblage is apparent even into later stages [7, 10]. Some phyllosphere taxa spend only a short part of their life cycle associated with the litter where they quickly sporulate and recolonize live plants [11], whereas others persist and are active throughout decomposition [2, 6]. Although less studied, bacteria from the phyllosphere can also be prevalent on decomposing leaves [5].

The role of the phyllosphere in leaf litter decomposition in aquatic systems has been largely unexplored (but see references below), despite the importance of leaf litter in stream food webs ([12]; and references therein). A meta-analysis reviewing the roles of plant endophytes in decomposition pointed to a dearth of studies in aquatic ecosystems [13] with the exception of three studies focusing on *Rhytisma* spp., fungal pathogens on leaves which can alter decomposition rates, and bacterial assemblages on submersed litter [14–16]. In streams, fungi are the primary decomposers on leaf litter based on biomass estimates, with bacteria increasing as decomposition progresses [17–20]. Microbial community structure on decomposing leaves in streams is driven by many factors including litter type, abiotic conditions and decomposition stage [19, 21–24]. Although long recognized as paraphyletic, aquatic fungi (classified as Ingoldian fungi or aquatic hyphomycetes) have primarily been studied in the domain of aquatic ecology [25–28]. Because taxonomy was largely based on spore morphology, which commonly occurs in the water for described aquatic hyphomycetes, ecologists underestimated the prevalence of aquatic hyphomycetes in terrestrial environments [29, 30]. Although there have been many reports of "aquatic" fungi distant from streams (e.g., [5, 30–32], or "terrestrial" fungi found in streams [22, 24], until recently these were considered the exception rather than the rule. DNA sequencing has confirmed that aquatic hyphomycetes can have distinct terrestrial life history stages [10, 30, 32, 33] with a recent study documenting that

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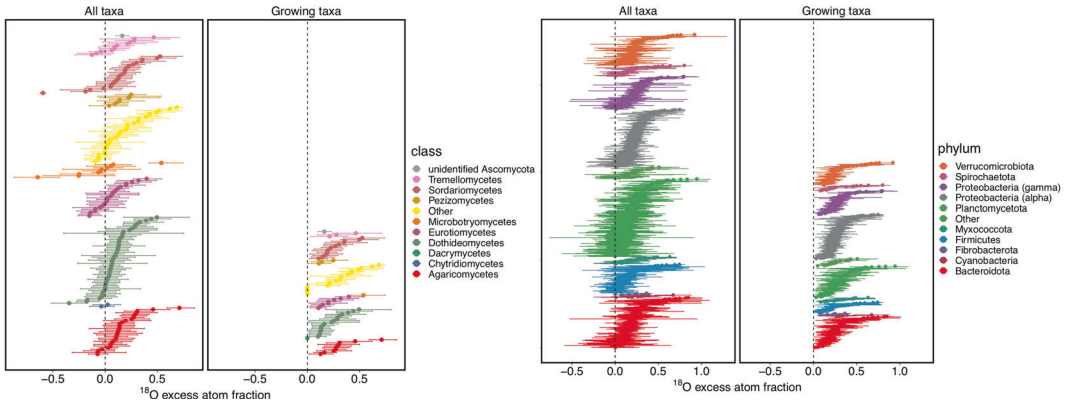


**Fig. 1 Comparisons of $^{18}O$ isotope incorporation into DNA for fungal species and bacterial ASVs found on submersed litter of Gambel Oak after 17 days.** All taxa were identified using 16S rRNA gene (bacteria) and ITS2 (fungi) sequencing. Growing taxa are the subset of total taxa based on 90% confidence intervals not overlapping with zero. Similar plots were generated for four different litter types harvested at days 17 and 24. This plot is representative of all treatments indicating that a higher proportion of bacterial ASVs were found growing on a given leaf type or harvest relative to the proportion of fungal species found growing.

approximately 65% of fungal species on submersed litter were also found as endophytes in terrestrial leaves [29]. These observations challenge the traditional view of "aquatic hyphomycetes" suggesting that many fungal taxa may have both terrestrial and aquatic life stages.

The sheer diversity of taxa uncovered by DNA sequencing makes it difficult to discern which species are functionally important. Because microbes can remain dormant for long periods, molecular based descriptions of community composition provide limited insight into the functional importance of species. Relative abundances of gene copies, sometimes used as a proxy for functional importance, are often not correlated with activity in freshwaters [34, 35]. For example, nearly 40% of the bacterial community in lake environments were inactive [34]. Here we employ quantitative stable isotope probing (qSIP), using water labeled with $^{18}O$ to differentiate members of the microbial community that are actively growing and reproducing from taxa that are present but not growing (Fig. 1; [36, 37]. Because $H_2O$ is a universal substrate for DNA replication [36–38] only cells that are dividing incorporate $^{18}O$ from $H_2{}^{18}O$. Using qSIP with $^{18}O$-labeled water estimates microbial growth, because organisms incorporate oxygen from water into their DNA during cellular replication, at rates proportional to their growth [36, 38–43]. By applying qSIP to analyze microbial assemblages on submersed litter, we can test whether microbial species that enter the river on dried leaves are growing during decomposition or are dormant once submersed. Comparing isotope incorporation across fungal and bacterial taxa throughout decomposition will test how growth of microbial taxa is affected by initial litter quality and varies as detritus is transformed.

This study addresses the following questions: (1) Does the growing microbial community on submersed litter enter with the leaf or colonize litter from the sediments and water column? (2) Does the composition of microbes entering with the leaves (i.e., the initial phyllosphere community) vary among leaf litter species collected from trees in the same watershed and is this initial imprint evident on decomposing litter? (3) How does the composition of growing microbes on submersed litter differ across plant species and through time? (4) Are response patterns similar for bacteria and fungi?

We hypothesized that the majority of growing microbes colonize litter in the water and that many of the microbes that enter with the leaf are transient once the litter is submersed. This hypothesis assumes that microbes with the highest growth rates on submersed litter complete their life cycles in the water. We also hypothesized that most of the fungal species would be growing, whereas most bacterial taxa would not be dividing. This is based on the prominent role of fungi in processing organic matter in streams and strong association between fungi and large organic particles [18, 44]. Because bacteria are abundant on many substrates (both organic and inorganic) and present in high numbers in the water column where they assimilate dissolved organic matter, we hypothesized that most bacteria passively colonize or settle on litter but remain dormant, particularly if they lack the enzymes to breakdown litter. We predicted that microbes entering with the leaf would differ across plant species and that these differences would be evident on submersed leaves early in decomposition. We also expected to see differences across plant species later in decomposition, but that these differences would be driven by different growth rates of microbes colonizing from the water [22, 23].

To test these hypotheses, we performed a decomposition experiment in laboratory mesocosms using stream water and sediments from Oak Creek, AZ. We characterized the microbial assemblages on dried leaf litter of four common riparian tree species using bacterial 16S rRNA gene and fungal ITS sequencing and used $^{18}O$-$H_2O$ qSIP to quantify the growth rates of bacteria and fungi on submersed litter at two time periods.

## METHODS

### Incubations

Methods follow those described in [37]. Senescent Arizona Ash (*Fraximus velutina*), Fremont Cottonwood (*Populus fremontii*), Narrowleaf Cottonwood (*Populus angustifolia*) and Gambel Oak (*Quercus gambelii*) leaves were collected from Oak Creek, AZ using bridal netting attached to tree limbs at least 10 meters from the river. These four species were chosen because they are common riparian trees and they differ in decomposition rates [45]. Ash and Fremont Cottonwood decompose quickly whereas Narrowleaf Cottonwood and Gamble Oak and decompose slowly [45]. Leaves were air dried and stored. In spring 2015, stream water and sediments were collected from Oak Creek, AZ. For each leaf type, four replicate freshwater mesocosms were constructed in 15 mL Falcon tubes that included 2 g of sediment, 9 mL of stream water and fifty 6 mm leaf disks (0.13–0.15 g). Mesocosms were incubated at room temperature in a shaker at 160 r.p.m. for 10 days to allow microorganisms to colonize the leaves [37, 46]. Stream water was replenished weekly. The lids of the Falcon tubes were left slightly unscrewed to allow for air exchange. After ten days, half of the Falcon tubes were centrifuged at 2250 × *g* for 10 minutes and the supernatant was removed. The stream water was replaced by 1 mL of

97 atom% $H_2{}^{18}O$ (Isoflex, San Francisco, CA) or sterile $H_2{}^{16}O$ water (0.2 atom% $^{18}O$), and the microcosms were incubated for an additional seven days and harvested at day 17. We repeated this process a week later for the second set of tubes which were incubated in paired $H_2{}^{16}O$ and $H_2{}^{18}O$ tubes from day 17- day 24. This design resulted in 64 samples total (4 replicates of the paired $H_2{}^{16}O$ and $H_2{}^{18}O$ incubations of the 17- and 24-day harvests for each leaf type). The $H_2{}^{16}O$ tubes serve as controls in qSIP experiments because they measure the density of taxa in unlabeled ambient water. We used these two time periods because it gave microbes time to colonize from the water column and sediments and allowed us to compare two time points when litter decomposition and microbial growth rates are high. Litter mass loss was measured after day 24, and an ANOVA and post-hoc Tukey's test were performed to compare the decomposition rates of the four leaf types.

### Density gradient centrifugation and gradient fraction

Genomic DNA was extracted from dried leaf disks to survey the resident phyllosphere microbial population, and the leaf disks incubated with water using a MoBio Powersoil Powerlyzer DNA extraction kit (Carlsbad, CA, USA) following the manufacturer's instructions with the addition of an initial 5 min incubation at 72 °C after the bead solution was added. To separate DNA by density, 1ug of DNA was added to a solution of cesium chloride and gradient buffer (0.1 M Tris, 0.1 M KCl, and 1 mM EDTA) in order to achieve a starting density of 1.70 g $mL^{-1}$ in 3.3 ml OptiSeal polyallomer tubes (Beckman Coulter, Brea, CA, USA). After centrifugation at 127,000 × *g* and 18 °C for 72 h using a Beckman TLN-100 rotor in an OptimaTM MAX ultracentrifuge (Beckman Coulter, Brea, CA), the gradient was separated into fifteen 200 µL fractions using a fraction recovery system (Beckman Coulter, Brea, CA). The density of each fraction was measured using a Reichert AR 200 handheld digital refractometer (Reichert Technologies, Buffalo, NY). The DNA present in each fraction was precipitated with isopropanol and resuspended in 50 µL of Tris-HCl buffer.

### 16S rRNA gene qPCR and sequencing

Standard curves were generated using 10-fold serial dilutions of genomic *Escherichia coli* DNA (ATTC, MG1655). The 10 µL reactions contained 0.2 µM of the primers 515F/806R [47], 0.01 U $\mu L^{-1}$ Phusion HotStart II Polymerase (Thermo Fisher Scientific, Waltham MA), 1× Phusion HF buffer (Thermo Fisher Scientific), 1.5 mM $MgCl_2$, 6% glycerol and 0.2 µm dNTPs. The assay was performed on a CFX 384 (Bio-Rad, Hercules, CA), using a program of 95 °C for 1 min followed by 40 cycles of 95 °C for 30 s, 64.5 °C for 30 s and 72 °C for 1 min. Bacterial gene copy numbers were calculated using a regression equation for each assay relating the cycle threshold (Ct) value to the known number of copies in the standards. For sequencing, two PCR steps were used [48]. Each sample was first amplified using primers 515 F and 806 R [47]. This was done in triplicate 10 µL PCR assays containing 1 µM of each primer, 0.01 U $\mu L^{-1}$, Phusion HotStart II Polymerase (Thermo Fisher Scientific), 1X Phusion HF buffer (Thermo Fisher Scientific), 3.0 mM $MgCl_2$, 6 % glycerol, and 200 µM dNTPs. PCR conditions were 95 °C for 2 min; 15 cycles of 95 °C for 30 s, 55 °C for 30 s, and 60 °C for 30 s. Initial PCR products were checked on a 1% agarose gel, pooled, tenfold diluted, and used as template in the subsequent reaction with region-specific primers that included the Illumina flow cell adapter sequences and a 12 nucleotide Golay barcode (15 cycles identical to initial amplification conditions). Products of the reaction were purified with carboxylated SeraMag Speed Beads (Sigma-Aldrich, St. Louis, MO) at a 1:1 v/v ratio as described in [49], and quantified by Picogreen fluorescence. Equal quantities of the reaction products were then pooled; the library was bead-purified once again, quantified by qPCR using the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA), and loaded at 11 pM (including a 30% PhiX control) onto a MiSeq instrument (Illumina, San Diego, CA) using 2 ×150 paired-end read chemistry.

### Bacterial data bioinformatic analysis

The paired-end reads were quality filtered, denoised, dereplicated, and chimera filtered using the DADA2 pipeline [50] within QIIME2 v2021.2 [51]. Taxonomy was assigned to the amplicon sequence variants (ASVs) using a trained Naïve Bayes classifier (SILVA 132) [52] for the 16S rRNA V3–V4 hyper variable region using the q2-feature-classifier plugin. Mitochondrial and chloroplast sequences were removed, and any ASVs that accounted for <0.05% of the total sequences were discarded [53]. Additionally, samples with fewer than 2,500 sequences were excluded from the analysis. The sequencing data from the dry leaves were rarefied at that depth.

### 18S rRNA gene qPCR and ITS2 sequencing

Standard curves were generated using genomic *Saccharomyces cereviciae* DNA (ATTC 201389d-5). The 10 µL reactions contained 1.25 µM of the primers FR1/FF390 [54], 1× QuantiTect SYBR Green PCR Master Mix (Thermo Fisher Scientific) and 2.5 mM $MgCl_2$. The assay was performed on a CFX 384 (Bio-Rad, Hercules, CA), using a program of 95 °C for 15 min followed by 35 cycles of 95 °C for 15 s, 50 °C for 30 s and 70 °C for 1 min. Fungal gene copy numbers were calculated using a regression equation for each assay relating the Ct value to the known number of copies in the standards. Sequencing library preparation was similar to constructing 16S rRNA gene sequencing libraries. The primers 5.8S-Fun and ITS4-Fun [55] were used in the two PCR steps in 10uL reactions containing 0.8 µM of each primer, 0.01 U $\mu L^{-1}$, Phusion HotStart II Polymerase (Thermo Fisher Scientific), 1X Phusion HF buffer (Thermo Fisher Scientific), 1.5 mM $MgCl_2$, 6 % glycerol, and 200 µM dNTPs. PCR conditions were 95 °C for 2 min; 20 cycles of 95 °C for 30 s, 55 °C for 30 s, and 60 °C for 1 min. Sequencing was carried out on a MiSeq Instrument using 2 ×250 paired-end read chemistry.

### Fungal data bioinformatic analysis

The paired-end reads were joined [56], fungal DNA sequences were extracted using ITSx [57], and sequences were analyzed with QIIME v 1.8 [58]. OTUs were picked using SWARM [59]. The most abundant sequence for each OTU was aligned with PyNAST [60] against the UNITE database [61], and taxonomy was assigned using RDP classifier [62]. The data were filtered to remove unassigned taxa at the kingdom level, and OTUs that made up less than 0.005% of all sequences, and samples with less than 400 sequences were excluded. The QIIME L7 species level OTU table was used for subsequent analyses.

### Data analysis

For a taxon to be included in subsequent analyses, it had to be present in three out of four replicates of the $^{16}O$ and $^{18}O$ incubations. The observed $^{18}O$ excess atom fraction (EAF) and 90% confidence intervals (CI) were calculated for each taxon according to Hungate et al. [36]. The weighted average density (WAD) was calculated for each taxon's DNA after incubation in the natural abundance and isotopically enriched water based on its distribution across a CsCl density gradient by summing the densities across all fractions multiplied by the total number of 16S rRNA gene or ITS copy numbers. The increase in weighted density in the enriched samples relative to the unlabeled treatments was calculated. We determined the GC content of the DNA for each taxon, based on its density, using the relationship of GC content and density based on a pure culture study [36]. The GC content was then used to calculate the molecular weights and the corresponding values of $^{18}O$ isotope composition for each taxon. Bootstrap resampling (with replacement, 1000 iterations) of replicates within each treatment was used to estimate taxon-specific 90% confidence intervals for the change in density and the corresponding value of $^{18}O$ EAF isotope composition [36, 37]. A taxon was considered to be $^{18}O$ labeled if the 90% CI was above the 0% EAF cutoff (Fig. 1). A Bray–Curtis similarity matrix of the relative abundance data was used for permutational multivariate analysis of variance (PERMANOVA) to compare community composition on leaf species at different time points. Relative abundances of fungal and bacterial taxa on the different leaf species were averaged by harvest. Averages were used for the regression analyses. To determine the proportion of the growing community, the relative abundances of the growers were summed. Taxa were considered residents if they were present on the dry leaves, and colonizers if they were only detected after the 17- or 24-day incubation.

All analyses were performed in Primer v6 [63] and R version 3.6 [64]. The qSIP code is publicly available at https://github.com/bramstone/qsip. Sequencing data can be accessed on NCBI SRA PRJNA 669386 (ITS2) and PRJNA 669516 (16S).

## RESULTS

In contrast to our predictions the majority of taxa were not growing although bacterial growth was more prevalent than fungal growth (Fig. 2A). Patterns were consistent across leaf types and harvests (Fig. 2A). On average, 42% percent of bacterial taxa divided during the total incubation period (range 28–59%) whereas on average only 16.5% of fungal species divided (range 7–27%). This pattern was also reflected in relative abundance

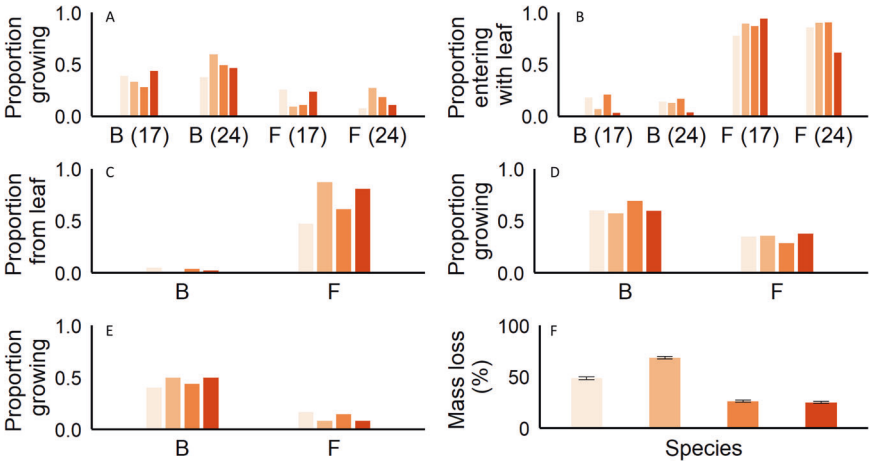


**Fig. 2 Comparisons of fungal (F) and bacterial (B) growth, relative abundance, and rates of decomposition among litter species. A** The number of taxa growing on decomposing leaves as a function of the total number of taxa on the decomposing leaves after 17 and 24 days. **B** The relative abundance of growing bacteria and fungi on submersed litter relative to the total abundance of bacteria and fungi after 17 and 24 days. Bacterial taxa and abundances were calculated based on 16S rRNA gene copy number and fungal species abundances were calculated based on ITS region copy number. **C** The proportion of growing taxa that entered on the leaves relative to the total number of growing taxa on each leaf type. **D** The number of growing taxa that entered on the leaves relative to the total number of taxa that entered on the leaves. **E** The number of growing taxa that colonized from the stream relative to the total number of taxa that colonized from the stream. **F** Differences in litter mass loss after 24 days of decomposition were significantly different. Error bars represent standard error.

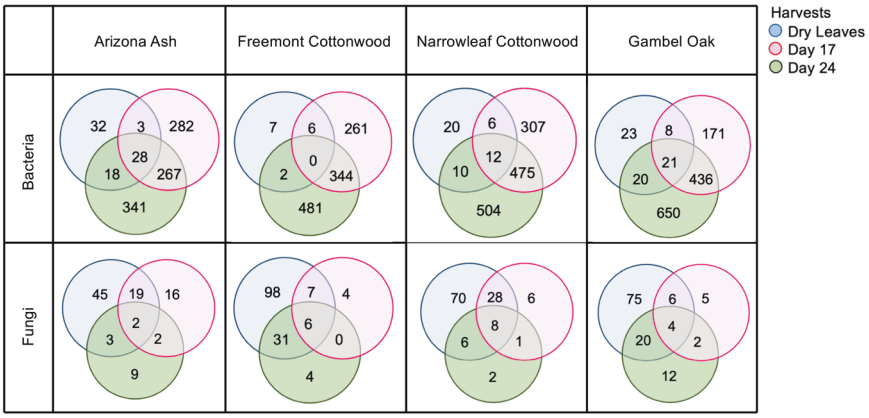


**Fig. 3 Venn diagrams of bacterial and fungal taxa that entered with the dried leaves and that were growing in submersed leaf litter after 17 or 24 days for four leaf species.** Diagrams include all taxa found on the dried leaves for each species, and all taxa that were growing in any of the individual replicates of the respective leaf species. Blue circles encompass taxa that entered with the leaves, pink circles encompass taxa growing on day 17 and green circles encompass taxa growing on day 24. Growing taxa were identified using qSIP.

where, on average across leaf types, the summed relative abundance of growing fungi was 52%, whereas the summed relative abundance of growing bacteria was greater than 70%. In contrast to our initial predictions, a higher proportion of fungal species entered with the leaves (Fig. 2B), though colonization by larger fungal spores may have been limited due to inoculum size. Consistent with our predictions, most bacterial taxa colonized litter in the water. The relative abundance of fungi that entered with the leaves averaged 79% and ranged from 56 to 96% (Fig. 2B). In contrast, the relative abundance of bacteria that entered with leaf averaged 12% and ranged from 3 to 21% (Fig. 2B). When focusing on the numbers of growing taxa, differences between bacteria and fungi are even more pronounced (Fig. 2C). On average 69% of the growing fungal taxa found on submersed litter entered with the leaf (Fig. 2C), in contrast only 3% of growing bacteria entered with the leaf. Together these results indicate that most growing fungi enter with the leaf while roughly a third colonize from the water column. On average only 33% of fungal taxa that entered with the leaf were growing suggesting that many endophytes do not replicate in aquatic environments (Fig. 2D). Although a small percentage of bacterial taxa entered with the leaves roughly half of those taxa were found growing (Fig. 2d). On average 46% of bacteria that colonized from the water column were found growing whereas only 12% of fungal species that colonized after submersion were growing.

Venn diagrams depict the numbers of taxa found on the dry leaves for each litter species and the numbers of growing taxa on submersed litter that were either residents (overlapping with $t = 0$ circles) or colonized after submersion (Fig. 3). The vast majority of growing bacterial species colonized the leaf after it was submersed. There were many fewer species of growing fungi and the majority of those found growing entered with the leaf (Fig. 3). There was also more overlap in growing species between the two harvests for bacteria than fungi (Fig. 3). Fungal species tended to be active at just one time point, with only between 4 and 10 species active at both harvests. The number of growing bacterial taxa increased with harvest date for all litter species indicating that more taxa colonize and grow as decomposition progresses. In contrast, similar numbers of fungal species were

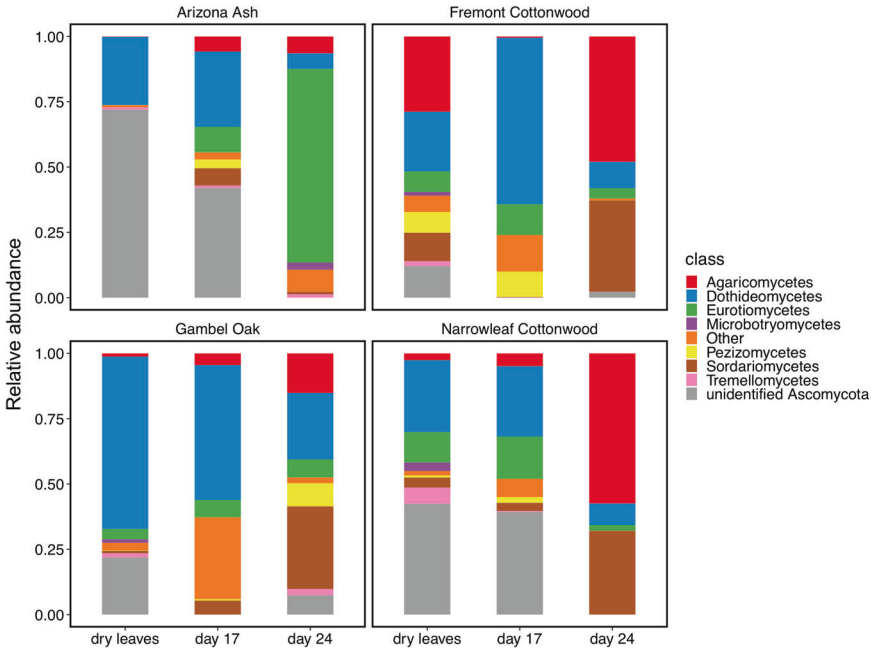


**Fig. 4 Relative abundances of fungal taxa found on dry leaves and growing on submersed litter of four leaf species harvested after 17 and 24 days.** Fungal community composition was significantly different across both leaf types and harvests.

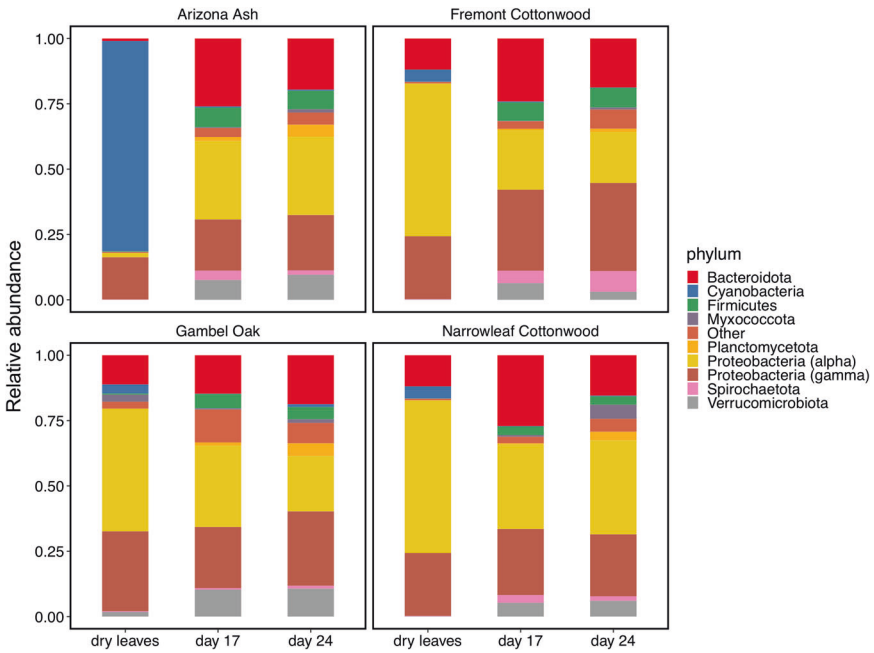


**Fig. 5 Relative abundances of bacterial taxa found on dry leaves and growing on submersed litter of four leaf species harvested after 17 and 24 days.** Bacterial community composition was significantly different across both leaf types and harvests.

active across harvest dates. Additionally, more of the fungal taxa entering with the leaf were active at day 24 than day 17, illustrating that phyllosphere taxa were not just growing in early decomposition stages, but could be dormant early in decomposition and growing at later stages. Most (>95%) bacterial and fungal taxa found on the dry leaves were also observed on the decomposing litter, although many were at very low relative abundances and did not grow.

Leaf types differed in decomposition rate, with Fremont Cottonwood and Arizona Ash decomposing more quickly than either Oak or Narrowleaf Cottonwood (Fig. 2F). As predicted, microbial assemblages differed across species and harvest dates (Figs. S1–S3). Initial leaf litter differed in both bacterial and fungal assemblages based on the relative abundances of taxa (Figs. S4 and S5). These assemblages also differed significantly across leaf types for fungi (Fig. 4, pseudo-$F = 6.4$, $p < 0.001$), and changed over time (harvest pseudo-$F = 8.4$, $p < 0.001$) depending on leaf type (interaction harvest × leaf: pseudo-$F = 8.4$, $p = 0.001$) PERMANOVA results also showed that the relative abundances of growing bacterial taxa differed across leaf types and over time (Fig. 5, pseudo-F: leaf = 7.6, harvest = 13.6, interaction = 6.9, all $p$ values < 0.001). Microbial assemblages did not group with decomposition rate nor were assemblages found on the two cottonwood species similar.

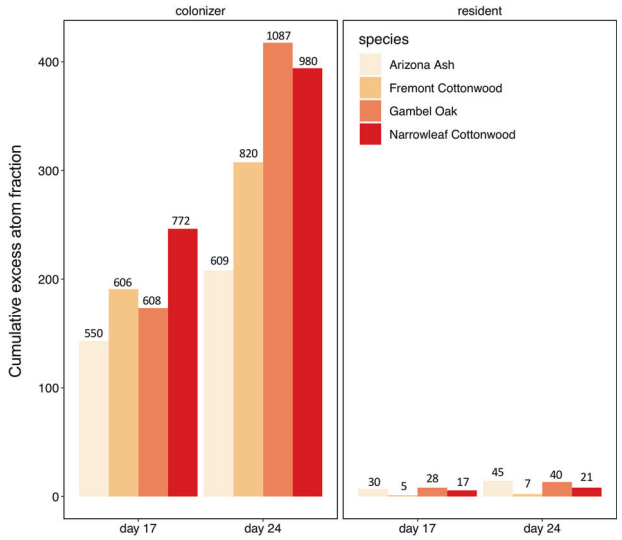


**Fig. 6 Cumulative excess atom fraction of $^{18}O$, an index of microbial growth rate, of the bacterial community growing on submersed leaf litter.** Higher values indicate more isotope incorporation and higher growth rates. Bacterial taxa are categorized by whether they colonized after leaves were submersed or were resident on dried leaves. Number of taxa in each category are shown above the bar (see Supplementary Table 1 for taxon-specific growth data). Results are presented for four leaf species and two harvest dates.

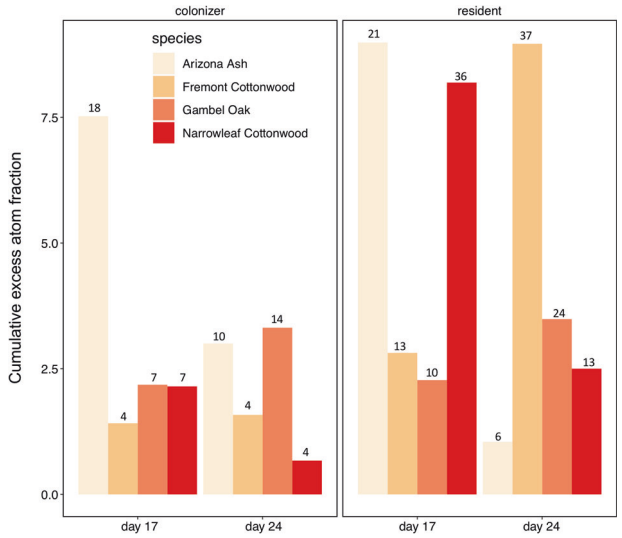


**Fig. 7 Cumulative excess atom fraction of $^{18}O$, an index of microbial growth rate, of the fungal community growing on submersed leaf litter.** Higher values indicate more isotope incorporation and higher growth rates. Fungal taxa are categorized by whether they colonized after leaves were submersed or were resident on dried leaves. Number of taxa in each category are shown above the bar (see Supplementary Table 1 for taxon-specific growth). Results are presented for four leaf species and two harvest dates.

Excess atom fraction, which measures the amount of $^{18}O$ incorporated in cells and is an index of growth rate, was higher for bacteria than fungi when summed across all growing taxa in each treatment, indicating that bacteria were replicating their DNA more rapidly than fungi (Figs. 6 and 7). Most of the $^{18}O$ isotope incorporation in bacteria was from taxa that colonized litter in the water (Fig. 6). Additionally, isotope incorporation into bacteria was higher in the second harvest indicating that bacteria grew more later in decomposition. In contrast, the isotope incorporation into fungal taxa was similar for colonizers and residents, except on Narrowleaf Cottonwood leaves at day 17 and Fremont Cottonwood leaves on day 24 (Fig. 7). Isotope incorporation by fungi was similar across harvests (Fig. 6). Although growth rates of individual fungal species changed through time, the overall $^{18}O$ incorporation of the entire fungal assemblages did not change with harvest date. Isotope incorporation into DNA was weakly correlated with relative abundances of bacteria ($R^2 = 0.07$, $p < 0.05$) and not correlated with relative abundance of fungi ($R^2 = 0.009$, $p = 0.14$).

The DNA of fungal class Agaricomycetes was highly enriched in $^{18}O$. Early and late colonizers of this class grew throughout the incubation and increased in abundance over time. Members of the Dothideomycetes were also highly enriched, but were more abundant after 17 than 24 days, indicating that growth for this class, that contains plant pathogens like *Passalora ampelopsidis*, occurred mainly during the first 17 days of the incubation. The resident *Cheatomium* sp. (class Sordariomycetes) was highly enriched after the 17-day incubation and made up 6–23% of the active community after 24 days (see Supplementary Table 1). The bacterial phyla Campilobacterota, Firmicutes, and members of the alpha- Proteobacteria, the majority of which colonized the leaves from the water column, were highly enriched across time points and leaf species. Several members of the order Bacteriodales were up to 90% enriched after 24 days, indicating that growing cells were not only incorporating the $^{18}O$ isotope from water, but also from nucleotides labeled with $^{18}O$ earlier in the incubation. The phylum Bacteroidales made up ~25 % of the growing community at both harvests in all the leaf species. Acidobacteriota, a common soil bacterium, and Actinobacteriota, one of the most taxonomically diverse bacterial phylum, both showed moderate levels of enrichment, while only contributing a small fraction of the total relative abundance. Verrucomicrobiota, a phylum often reported to increase in relative abundance later in decomposition, showed slight increases in enrichment between harvest dates (see Supplementary Table 1).

## DISCUSSION

Subsidies between terrestrial and aquatic ecosystems are important in maintaining diversity and productivity in both ecosystems [65, 66]. When leaves fall in rivers, they are more than packets of dead organic matter. Leaves bring with them suites of microbes that contribute to their breakdown. Microbes are more nutritious for aquatic invertebrates than dead plant material and those that enter with leaves likely contribute substantially to diets of aquatic invertebrates [12]. Our results combined with others establishes that many aquatic fungi are also plant endophytes [29, 30, 32, 33]. This may prove to be an important life history pattern of many aquatic fungi that may regularly alternate between terrestrial and aquatic ecosystems. Further, we show that some fungi, previously thought to only thrive in terrestrial systems, can grow and divide in aquatic ecosystems [29]. For example, members of the fungal class Glomeromycota, a root symbiont, grew throughout decomposition of submersed litter and across leaf types (see Supplementary Table S1).

We observed, as other terrestrial studies have, that fungal taxa associated with the phyllosphere constitute a significant share of the fungal community during leaf decomposition [4, 67]. Similar to our results, fungi from the phyllosphere were more active (inferred from relative RNA abundances) than those colonizing litter from soil [10]. Over the two time periods that we sampled, the majority of fungi did not grow, with different taxa growing between the two harvests, indicating that fungi remain dormant until conditions are conducive to their growth. Fungal species that entered with the leaf only grew for a short time period, perhaps capitalizing on specific decomposition stages and indicating

temporal niche partitioning among fungal species based on their suites of enzymes capable of degrading litter constituents [6]. Because we only measured two time periods, our results likely underestimate the number of species that grew. Sampling multiple times beginning when the leaf enters the river until mass loss is complete will provide a more comprehensive perspective of how many and when certain fungal species grow. It is difficult to mimic field conditions perfectly in the laboratory. Mesocosms in this experiment lack the continuously replenishing flows inherent to stream ecosystems and the expense of labeled water precludes using it in flow through artificial streams. As a result, nutrient availability can be reduced during lab conditions. Aquatic microorganisms and rates of leaf litter decomposition are sensitive to changes in nutrient concentrations [68, 69]. To minimize artifacts mesocosms were kept on shaker tables and fresh stream water was added each week. We found that mass loss was similar to field studies for these plant species, indicating that microbial activity was similar [70]. It is possible that the number of microbial species that entered from the water column was lower in this lab incubation although the proportion of fungi that entered with the leaf was similar to observations from a field study [29]. In addition, the total numbers of bacterial and fungal taxa were similar to field incubations [70]. Despite experimental artifacts, this study presents, for the first time, taxon-specific growth rates during aquatic decomposition and provides testable hypotheses for subsequent experiments which can be extended to the field. Incubating leaves in situ and then submersing them in labeled water for a short period of time to label growing organisms will also decrease incubation time in an artificial environment. As we refine protocols for qSIP we will be able to sample more extensively and for shorter incubation periods to delineate more precisely when microbial species are producing new cells. Additional sampling points combined with in situ incubations will capture successional dynamics associated with leaf litter decomposition, as microbial assemblages change significantly during the initial stages of decomposition [22, 24], and early samples might reveal growth of significantly more phyllosphere fungi.

This study also demonstrates differences in litter colonization and growth between bacteria and fungi. Most bacterial taxa colonized decomposing leaves in the water and formed new cells. Many bacterial taxa that grew prior to the first harvest also grew by the second harvest with increasing numbers of newly formed bacterial cells through time. Bacterial assemblages tend to differ with time in the river [23, 71], which was primarily due to shifts in relative abundances of different species through time. We also observed increased growth rates of bacteria at the second harvest, which is consistent with patterns observed in both aquatic and terrestrial ecosystems in which the dominance of bacteria increases as decomposition progresses [71, 72]. Quantitative SIP provides unique measurements of microbial population dynamics by measuring taxon-specific growth in intact microbial assemblages. Combining this technique with traditional approaches such as ergosterol and enzyme assays to assess microbial biomass and activity, will allow us to scale abundance and growth of individual species to ecosystem processes.

Initial differences in microbiomes across plant species were retained in the aquatic environment and played a role in structuring the microbiome on decomposing litter particularly for fungi. Differences in microbial assemblages across leaf species are driven by multiple interactions including priority effects of endophytes and differences in initial phytochemistry [23, 70]. In terrestrial soils, comparisons of sterilized and non-sterilized leaves indicate that fungal endophytes affect fungal community composition but not decomposition [73]. Future studies comparing sterilized and non-sterilized leaves [73] will provide insight into the magnitude of each effect. Future research comparing growing microbes from the same litter in terrestrial soils and nearby streams could test for the breadth of ecological roles that endophytes can play in decomposition across ecosystem boundaries.

## CONCLUSION

This study advances the field by demonstrating that most of the growing fungi on submersed leaves have a terrestrial life stage and enter the water with the leaf. This research confirms and expands upon observations of the prevalence of terrestrial fungi on submersed litter [29]. Fungal species are more responsive to stages of decomposition than bacteria and switch from dormancy to growth as conditions change. In contrast, most bacteria colonized from the water column with the number of actively dividing species increasing with time. Bacteria were less sensitive to decomposition stage and grew regardless of litter type. Molecular databases for aquatic microbes are increasing rapidly [74] and will be instrumental in discerning the ecological interactions between microbes in different environments. Future studies using isotope-enabled genomics will allow ecologists to understand how microbiomes transcend ecosystem boundaries and impact biogeochemical cycling in both terrestrial and aquatic ecosystems.

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## AUTHOR CONTRIBUTIONS

All authors contributed to the conceptual development of this work. JCM, BAH, ES, and MH designed the incubation study. AAS and MH processed the samples. MH performed molecular analyses and analyzed the data with contributions from JCM, ASW and BJK. JCM, ASW, MH, and BAH wrote the manuscript, with editorial contributions from all authors.

## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

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