Vol. 83: 237–250, 2019
https://doi.org/10.3354/ame01916

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online October 17

Succession of bacteria and fungi in leaf litter of tree hole habitats: responses of diversity to mosquito larvae

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ABSTRACT: The diversity of leaf-associated microbial communities is predicted to show a unimodal pattern over time, with peak taxonomic diversity occurring during mid-decomposition. Predation may modify successional trajectories, with the nature of modifications depending on the stage of community development. We compared temporal changes of early-stage and established bacterial and fungal communities associated with senesced American beech (*Fagus grandifolia*) leaves with and without predation by *Aedes triseriatus* mosquito larvae. Bacterial and fungal communities had different successional patterns. Established bacterial communities were more diverse compared to those in the early stages of succession, while fungal diversity was similar in both developmental stages. Fungal diversity generally remained constant while bacterial diversity showed a non-linear, unimodal pattern over time. Community composition differed significantly between early-stage and established communities for both bacteria and fungi; compositional shifts over time were more marked in early-stage communities for both groups. Predatory effects depended on the stage of community development and community type. In early-stage communities, predation increased bacterial richness; however, richness in established communities decreased with predation. The contrasting successional trajectories of bacterial and fungal communities suggest differential successional drivers and responses to predation for microorganisms inhabiting a shared substrate.

KEY WORDS: Microbial succession · Leaf-associated microbes · Detritivory · *Aedes triseriatus* · Tree holes

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1. INTRODUCTION

How and why biological communities change over time has long been of interest to ecologists, with much attention given to succession of plant communities (Pickett et al. 1987, Meiners et al. 2015, Pulsford et al. 2016). Microbial communities are unique in many ways, including their extraordinary phylogenetic and metabolic diversity (Sogin et al. 2006), the frequency of dormant behavior (Lennon & Jones 2011), and short generation times, all of which may influence successional dynamics. Fierer et al. (2010) considered microbes within the classic plant successional framework. They proposed that while similar processes drive plant and microbial succession (e.g. dispersal, resource limitation, species interactions), temporal patterns of microbial diversity depend upon microbial metabolic strategies and type of substrate being colonized. For example, endogenous heterotrophic microbial communities (sensu Fierer et al. 2010), such as those that colonize detritus, metabolize substrate carbon and change the quality of their substrate over the course of succession. Fierer et al. (2010) predicted that the diversity of endogenous heterotrophic microbial communities will increase, peak, and decline during initial, intermediate, and later stages of succession, respectively, corresponding with carbon dynamics.

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Successional trajectories can be modified by environmental context, including top-down pressures. Herbivory can strongly influence plant communities (Carson & Root 1999, Van Moorleghem & de la Peña 2016, Alberti et al. 2017) depending on evolutionary history and contributing environmental factors (Olff & Ritchie 1998); thus, the stage of succession when herbivory is applied can influence plant community response (Davidson 1993, Milchunas & Vandever 2014). For example, whether herbivores positively or negatively affect the rate of succession depends on if and when (i.e. early or late succession) they consume dominant or keystone species (Carson & Root 1999, Olnes & Kielland 2016) or if they target early-, mid-, or late-successional species (Davidson 1993).

Our understanding of how top-down factors influence the succession of heterotrophic microbial communities has developed slowly, partly due to challenges associated with quantifying microbial diversity and describing microbial community composition. However, an example of a system in which top-down effects on microbial communities have been relatively well-studied is headwater streams, where microbes associated with leaf litter are consumed by a variety of macroinvertebrate detritivores (e.g. Cummins 1973). The impact of predation on these communities is often assessed by comparing communities protected from, and subjected to, detritivores (e.g. leaf packs constructed with differing mesh sizes). Such studies have shown positive, neutral, and negative fungal diversity responses (Bärlocher 1980, Rossi 1985, Ferreira & Graca 2006, Mora-Gómez et al. 2016) and shifts in fungal and bacterial composition (Kaufman et al. 1999, 2008, Walker et al. 2010, Moghadam & Zimmer 2014, 2016, Mora-Gómez et al. 2016). However, investigations comparing protected and exposed communities tend not to consider how the stage of community development may alter top-down effects.

The resilience of microbial communities at different stages of development to predation may have important implications for the structure and function of these communities, particularly in habitats that contain detritus in various stages of microbial colonization. Water-filled tree holes are cavities formed by tree root buttresses that retain precipitation. In temperate areas, these habitats collect leaves and other detritus throughout the year, with a large input of senescent leaves in the autumn. Due to the lack of lateral flow and the absence of shredding macroinvertebrates, tree holes are more retentive of leaf material than headwater streams, where shredders can process a substantial proportion of leaf litter (Cuffney et al. 1990). These conditions, along with inputs of fresh leaves, create detritus pools spanning a range of microbial colonization. These habitats contain several detritivores, the most abundant being mosquito larvae, which consume waterborne and leaf-associated microbes (Kaufman et al. 1999, 2001, 2008).

Our study had 2 objectives: to quantify successional changes in leaf-associated microbial community structure and to compare the effects of predators on early-stage and established microbial communities. We used the water-filled tree hole and eastern tree hole mosquito *Aedes triseriatus* larvae (a common inhabitant of this habitat) as a model study system to address the following hypotheses:

Hypothesis 1: Endogenous heterotrophic microbial community diversity peaks during the intermediate stages of succession (Fierer et al. 2010). Therefore, we predicted that early stage communities would be less diverse than older, established communities (Prediction 1) and that diversity would be unimodal over time (Prediction 2).

Hypothesis 2: Predation can increase diversity by reducing competition (Terborgh 2015). However, we predicted that this positive effect would depend on the stage of community development and that predation on communities in early stages of development would prevent new taxa from colonizing, keeping diversity low over time (Prediction 3).

We tested these predictions on 2 types of microbial communities: the community loosely associated with leaf litter and the community tightly adhered to leaf surfaces. Mosquito larvae have a browsing feeding mode which may not effectively harvest tightly adhered cells. Therefore, we predicted that mosquito larvae would have a greater effect on surficial microbial communities compared to those tightly associated with leaf litter. Fierer et al. (2010) did not distinguish between successional trends of bacterial and fungal endogenous heterotrophic communities, but the fact that fungal hyphal cells are usually embedded within leaf material may lead to differential mosquito impacts.

2. MATERIALS AND METHODS

2.1. Experimental design and sampling

We used mesocosms constructed from lengths of opaque PVC pipe capped on one end and pipe insulation glued to the outsides to simulate tree-hole habitats. Each mesocosm initially contained 300 ml of deionized water, 3 ml of homogenized water col-

lected from several natural tree holes found at the base of American beech (*Fagus grandifolia*) trees in order to introduce a natural microbial community, and dried beech leaves (petioles removed). Neither the water nor the leaves were sterilized prior to addition, so mesocosms contained terrestrial microbial communities associated with the leaves, any microbes contained within the deionized water, as well as the natural aquatic microbial community found in the tree hole inoculum.

This study occurred concurrently with an investigation of the effects of leaf condition on *Aedes triseriatus* production (Norman & Walker 2018). The full experiment was a 2×2×3 factorial design with 2 conditioning periods (3 d and 1 mo), 2 leaf rations (1 and 2 g), and 3 larval densities (0, 30, or 40 larvae per mesocosm) with 6 replicates per treatment. However, for this study we used the following subset: 2 conditioning periods (3 d and 1 mo), 1 leaf ration (1 g), and 2 larval densities (0 and 30 larvae per mesocosm) and randomly selected 3 of the 6 replicates to sample for microbial community analysis.

Leaves were conditioned for 3 d or 1 mo to create early-stage and established microbial communities, respectively. Mesocosms were covered with black mesh (~1 mm pore size) and placed in a 25°C incubator with a 12/12 h light/dark cycle during the conditioning period. The timing of leaf submersion was staggered to allow both sets to finish conditioning on the same day. Following the condition period, a total of 15 leaf disks (1.5 cm diameter) were cut from several leaves selected from each mesocosm. We sampled 2 microbial communities from these leaf disks: the community loosely adhered to the leaf surface and the community firmly imbedded within the leaf substrate. We defined these groups operationally following Kaufman et al. (2008), who published some of the first detailed descriptions of tree hole microbe communities. We sonicated 3 groups of 5 leaf disks in 2 ml of sterile phosphorus buffer solution for 12 min and centrifuged the sonicate in order to pelletize the microbial cells. These pellets contained the microbes easily dislodged from the leaves, or what we refer to as the loosely adhered community. The microbes still attached to the leaves following sonication were what we refer to as the firmly imbedded community. Pellets and sonicated leaf disks were frozen at −80°C.

At this point, the water was carefully drained and replaced with 300 ml of beech stemflow in order to create similar water quality conditions across the treatments. Thirty newly hatched *A. triseriatus* larvae were added to half of the mesocosms from each conditioning treatment. Larvae were hatched from a laboratory colony maintained at Michigan State University (Walker 2016). Mesocosms were incubated under the same temperature and light regime as during the conditioning period. Stemflow was added periodically to replace volume lost to evaporation. Stemflow additions were undoubtedly a source of microbial colonization, but these additions were not correlated with any treatment and mesocosms were not closed systems. Blowers within the incubators likely contributed to dispersal of airborne microbes among all mesocosms. Adult collection procedures and mosquito responses are described by Norman & Walker (2018).

Individual mesocosms were sampled for microbial communities as previously described when either less than 10% of the larvae remained or when no pupae had been produced in more than 7 d. Mosquitoes developed at different rates depending on condition, ration, and density (Norman & Walker 2018), causing mesocosms to be sampled on different dates (see Table S1 in the Supplement at www.int-res.com/articles/suppl/a083p237_supp.pdf). However, mesocosms within the same larvae treatment were sampled within 11 d of each other.

2.2. Microbial community analysis

For clarity, we refer to loosely adhered and imbedded communities as community types, communities with 3 d and 1 mo conditioning times as 3D and 1M communities, and communities sampled from mesocosms immediately following the conditioning period, with larvae, and without larvae as 'initial,' 'larvae,' and 'no larvae' communities, respectively.

Nucleic acids were extracted from sonicated leaf disks and sonicate pellets using PowerSoil Powerlyzer extraction kits (Qiagen) according to the manufacturer's specifications, and frozen at −80°C. Pellets from the 3 sonicate subsamples (each composed of sonicate from 5 leaf disks) collected from a single mesocosm were pooled and extracted as a single sample. Sonicated leaf disks were similarly pooled.

Amplicon libraries for bacterial community diversity analysis were constructed via direct amplification of the V4 region of the bacterial/archaeal small subunit ribosomal RNA gene using primer set 515F (GTG CCA GCM GCC GCG GTA A) and 806R (GGA CTA CHV GGG TWT CTA AT) (Caporaso et al. 2012, Kozich et al. 2013). Pooled amplicons were sequenced using an Illumina MiSeq flow cell in a 2 × 250 bp paired-end format. Base calling, demulti-

plexing, and FastQ conversion were accomplished using Illumina Real Time Analysis (v. 1.18.54) and Illumina Bcl2fastq (v. 1.8.4). Bacterial community sequence analysis was done using the QIIME bioinformatics pipeline (v. 1.9.1; Caporaso et al. 2010a) on Amazon's EC2 cloud computing resource with the qiime-191 AMI. Sequences were trimmed using TRIMMOMATIC (v. 0.33; Bolger et al. 2014), and sequence quality was assessed using FastQC (v. 0.11.3; Andrews 2010). Paired-end reads were merged using PANDAseq (v. 0.16.1; Masella et al. 2012). An 'open reference' method was used to cluster sequences using 'usearch61,' which included chimera checks and quality filtering using UCHIME (Edgar 2010, Edgar et al. 2011) with Greengenes as the reference database (McDonald et al. 2012). A 97% similarity threshold was used to define operational taxonomic units (OTUs). PyNAST (v. 1.2.2; Caporaso et al. 2010b) and uclust (Edgar 2010) were used to align sequences and assign taxonomy, respectively. Singletons were removed.

Amplicon libraries for fungal community diversity analysis were constructed using a dual PCR strategy and primer set ITS1FI2 (GAA CCW GCG GAR GGA TCA) and ITS2 (GCT GCG TTC TTC ATC GAT GC) (Schmidt et al. 2013). Sequencing, base calling, demultiplexing, and FastQ conversion processes were similar to those described for 16s amplicons. Fungal internal transcribed spacer (ITS) sequences were processed using PIPITS (v. 1.3.4; Gweon et al. 2015; https://github.com/hsgweon/pipits), an open source pipeline developed for analysis of fungal communities from MiSeq data. PEAR (v. 0.9.6; Zhang et al. 2014) was used to join read pairs, and FASTX-Toolkit (v. 0.0.14; http://hannonlab.cshl.edu/fastx_toolkit/) was used for quality filtering. The ITS1 subunit was extracted using ITSx (v. 1.0.11; Bengtsson-Palme et al. 2013). OTU clustering was performed using VSEARCH (v. 1.11.2; Rognes et al. 2016), following removal of short sequences (<100 bp) and singletons. OTUs were defined with a 97% similarity threshold. Chimeras were removed using a retrained UNITE UCHIME reference database (http://sourceforge.net/projects/pipits/files/UNITE_retrained_31.01.2016.tar.gz, https://unite.ut.ee/sh_files/uchime_reference_dataset_01.01.2016.zip; Gweon et al. 2015), and taxonomy was assigned using the RDP Classifier (v. 2.9; Wang et al. 2007). Fungal ITS sequences were processed using the High Performance Computing Center at the Institute for Cyber-Enabled Research (iCER) at Michigan State University.

2.3. Data analysis

Bacterial and fungal sequences are deposited in the NCBI Sequence Read Archive, BioProject number PRJNA507902.

We analyzed alpha diversity by estimating the richness (number of OTUs) of each replicate. Bacterial and fungal richness were calculated using the alpha_diversity.py qiime script and the diversity function in the R package 'vegan' (v. 2.4-3; Oksanen et al. 2017), respectively. Richness was calculated following rarefication to a consistent number of sequences per sample. The average richness of 3D and 1M initial communities were compared using a Student's *t*-test.

Changes in bacterial richness over time in treatments without larvae were modeled using second-degree polynomial regression with the total number of days of incubation (including the initial conditioning period) as the measure of time. This equation was used to predict bacterial richness in communities without larvae on each day a mesocosm with larvae was sampled. We calculated change in richness in early-stage communities with predation over time (average of 27 d) by subtracting the average richness of the 3D/initial communities from the richness of each 3D/with larvae replicate. Similarly, we calculated change in richness in early-stage communities without predation as the difference between average richness of the 3D/initial communities and the richness predicted from the polynomial regression described above. We compared the change in richness between early-stage communities with and without larvae using a Student's *t*-test. The impact of larvae added to established bacterial communities was assessed similarly using 1M/initial communities.

Fungal richness did not change predictably over time. Therefore, the impacts of larvae on fungal richness were assessed as described above using the observed richness of no larvae treatments collected as close in time to the larvae treatments as possible rather than predicted values.

Community composition was compared across treatments using Bray-Curtis dissimilarity matrices and visualized using principal coordinate analyses (PCO) and ordination. Dissimilarity matrices were created from rarefied OTU tables using the 'vegdist' function in the R package 'vegan' (v. 2.4-3; Oksanen et al. 2017). PCO were done using the 'pcoa' function in the R package 'ape' (v. 4.1; Paradis et al. 2004). Shifts in relative abundance of individual taxa between treatments were assessed using Student's *t*-tests.

Permutational multivariate ANOVA (PERMANOVA) tests were used to evaluate individual predictions for bacterial and fungal communities. The effects of stage of community development (Prediction 1) were tested using a 2-way PERMANOVA with community type (loosely adhered and imbedded) and stage of community development (3D and 1M) as factors. Changes in community composition over time without larvae (Prediction 2) were assessed using a 3-way PERMANOVA with community type, stage of community development, and manipulation (initial and no larvae communities) as factors. Changes in community composition over time with larvae were assessed similarly with initial and larvae communities as levels of the manipulation factor. PERMANOVA tests were done using the 'adonis' function in the R package 'vegan' (v. 2.4-3; Oksanen et al. 2017).

3. RESULTS

We obtained and processed 16s rRNA gene and ITS sequence data for the original 2×3×3 factorial experiment (3 of 6 replicates), a total of 96 samples. A total of 3 357 341 paired-end bacterial 16s sequences were retained following trimming, of which 1 516 861 were successfully merged and aligned. We excluded 5 samples from further analysis due to low sequence numbers (≤635). We subsampled each community to a depth of 4755 sequences (the minimum number of sequences found in the remaining samples). The final bacterial community dataset contained 92 samples and 32 450 OTUs. Fungal ITS sequencing yielded a total of 2 293 835 pairs of reads with 1 996 202 containing the ITS1 region and 1 971 795 retained following chimera and quality checks. The final fungal community dataset was rarefied to 11 128 sequences per sample and contained 2041 OTUs with 302 phylotypes. We selected the samples relevant to this study from the final datasets (2 conditioning periods, 1 g leaf ration, and 0 and 30 larvae per mesocosm) simplifying the original 2×2×3 design to a 2×1×2 design with each treatment consisting of the 3 randomly selected replicates). All further descriptions apply to this subset.

Bacterial communities were dominated by *Proteobacteria* (44–90% relative abundance), with *Bacteroidetes* well represented in most communities (9–38% relative abundance; Fig. 1). Individual communities were dominated by relatively few taxa, with the 3 most abundant OTUs comprising 24–96% of the total abundance (Table S2).

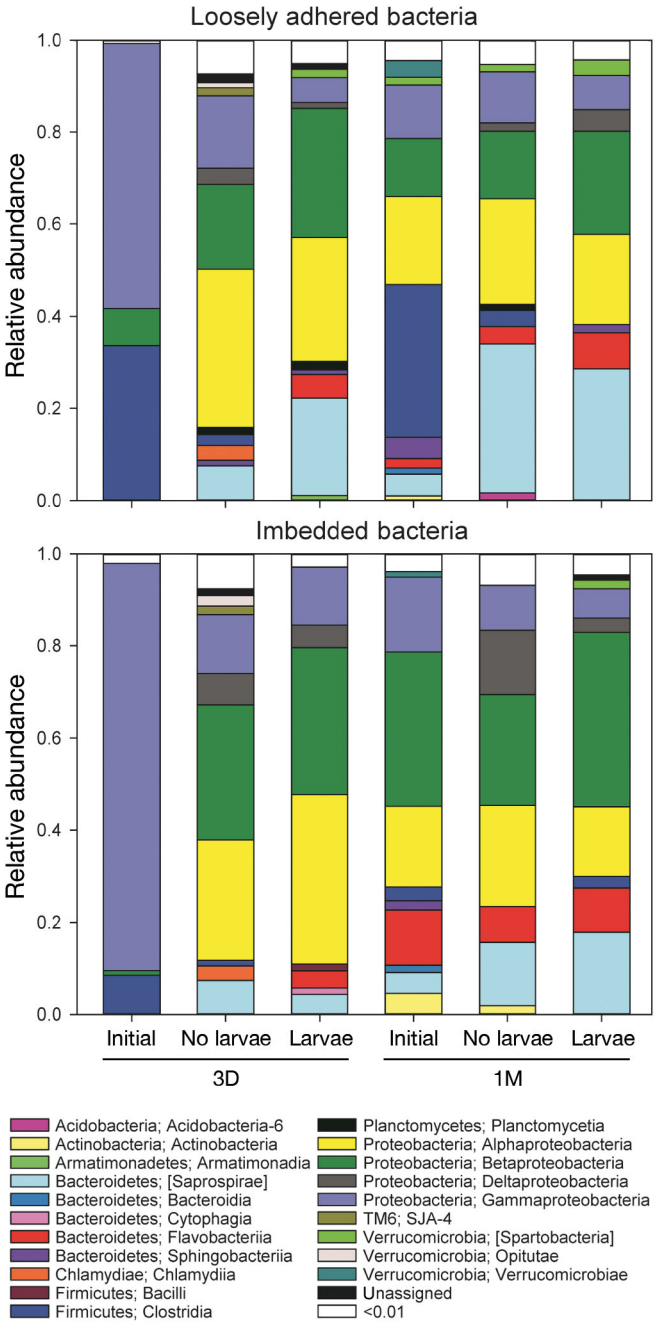

Fig. 1. Mean relative abundance of bacterial classes in (A) loosely adhered and (B) imbedded communities across treatments. Classes with <1% relative abundance were grouped into a single category

Fungal communities were dominated by Ascomycota (32–89% relative abundance) with Basidiomycota found in varying relative abundances (<1–36%; Fig. 2). A substantial proportion of the ITS sequences were classified as unknown fungi (10–62% relative abundance) or were unassigned (<1–25% relative abundance). Fungal taxa were generally distributed

more evenly than bacterial communities, with the 3 most abundant fungal OTUs comprising 25–63% of the total fungal abundance (Table S3).

3.1. Early-stage vs. established communities (Prediction 1)

3.1.1. Bacterial communities

Early-stage and established bacterial communities differed from each other in both community types. Communities contained >600 more bacterial OTUs after 1 mo (1M) than after 3 d (3D) in both the loosely adhered and imbedded communities ($p < 0.042$; Fig. 3). 3D and 1M initial bacterial community composition differed from each other for both community types (Fig. 4A, Table S4). *Proteobacteria* were generally more abundant in 3D compared to 1M initial communities for both loosely adhered and imbedded community types, although this difference was only significant in imbedded communities ($p = 0.03$; Fig. 1A). *Gammaproteobacteria* made up a significantly greater proportion of *Proteobacteria* in 3D/initial communities compared to 1M/initial communities in both community types ($p < 0.001$), while *Alphaproteobacteria* showed the opposite pattern (<1% compared to 44%; $p \leq 0.05$). The relative abundance of *Bacteroidetes* was greater in 1M/initial compared to 3D/initial imbedded communities only ($p = 0.002$), and *Verrucomicrobia* and *Firmicutes* were similar between 3D and 1M initial communities of both types ($p > 0.08$).

3.1.2. Fungal communities

Fungal richness responded differently than bacterial richness during the conditioning period. Fungal richness was similar between early-stage and established communities of both types ($p > 0.149$; Fig. 5A,B). Fungal community composition differed between 3D and 1M initial communities for both community types (Fig. 4B, Table S5). Ascomycota relative abundance was generally, although not significantly, lower in 1M/initial compared to 3D/initial communities of both types ($p \geq 0.339$; Fig. 2). Ascomycota were predominantly Dothideomycetes or unassigned Ascomycota. Dothideomycetes made up a smaller proportion of Ascomycota in 1M/initial communities than in 3D/initial communities ($p \leq 0.041$). The relative abundances of Basiodiomycota, unknown fungi, and unassigned taxa were similar between 3D/initial and 1M/initial communities ($p > 0.119$).

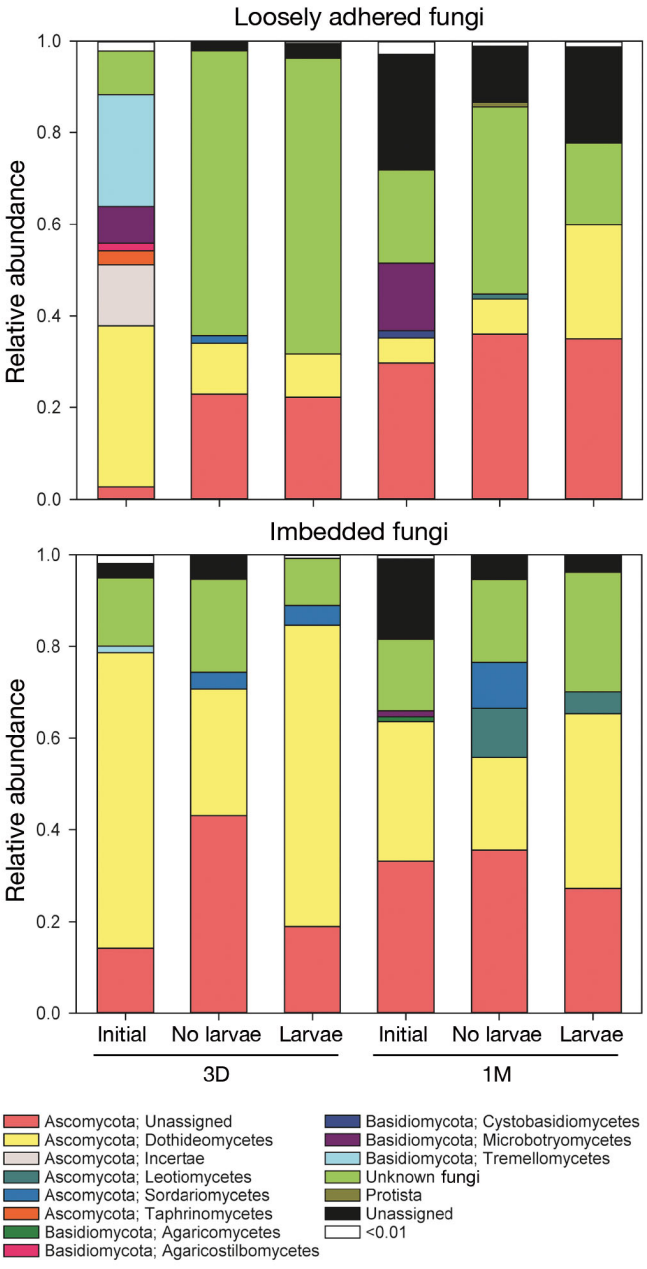

Fig. 2. Mean relative abundance of fungal classes in (A) loosely adhered and (B) imbedded communities across treatments. Classes with <1% relative abundance were grouped into a single category

3.2. Community changes over succession (Prediction 2)

3.2.1. Bacterial communities

Bacterial richness changed in a unimodal pattern over time (Fig. 3C,D). Loosely adhered and imbedded communities contained an average of 970 OTUs after 51 d (3D/no larvae treatments) compared to an

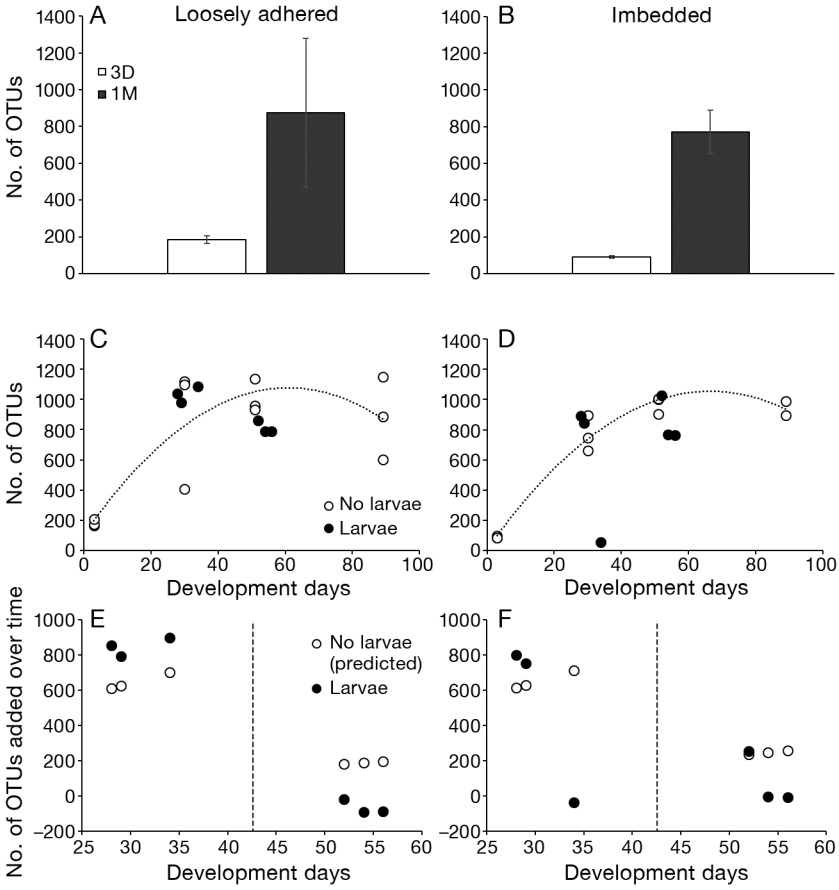

Fig. 3. Assessment of predicted changes in (A,C,E) loosely adhered and (B,D,F) imbedded bacterial richness. Bars in (A) and (B) compare mean (±SD) richness of 3 d (3D) and 1 mo (1M) initial communities (Prediction 1; see Section 1 for details of predictions). (C) and (D) describe changes in richness over time (Prediction 2). Dotted lines are second-degree polynomial regressions (C: $y = -0.26x^2 + 31.9x + 105.2$, $R^2 = 0.64$, $p = 0.004$; D: $y = -0.23x^2 + 31.5x + 7.63$, $R^2 = 0.96$, $p < 0.001$). (E) and (F) compare changes in richness over time in communities exposed to mosquito larvae and communities without larvae as predicted from the regressions shown in (C) and (D) (Prediction 3). Symbols to the left of vertical lines denote changes in richness in early-stage communities (e.g. 3D/larvae – 3D/initial) and symbols to the right denote changes in richness in established communities (e.g. 1M/larvae – 1M/initial).

average of 91 OTUs after 3 d (3D initial treatments), with slight declines in average richness by the end of the 89 d experiment. Early-stage and established bacterial communities differed in how much community composition changed over time (Fig. 4A). 3D/initial communities were more dissimilar from the 3D/no larvae communities than 1M/initial communities were from 1M/no larvae communities, as indicated by a significant condition × manipulation interaction (Table S4). This pattern occurred in both loosely adhered and imbedded community types.

Temporal shifts in composition of early-stage communities were characterized by a general, but not significant, decrease in the relative abundance of *Firmicutes* as well as an increase in the relative abundances of *Bacteroidetes* and *Verrucomicrobia* ($p \leq 0.032$; Fig. 1). There were also marked shifts in class distribution within *Proteobacteria*, including a decrease in the proportion of *Gammaproteobacteria* over time ($p \leq 0.001$) and corresponding increases in the proportion of *Alpha-* and *Deltaproteobacteria* in loosely adhered communities ($p \leq 0.016$) and *Alpha-* and *Betaproteobacteria* in imbedded communities ($p \leq 0.042$). Temporal shifts in the composition of established communities were less dramatic. Relative abundances of *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* did not change significantly over time ($p > 0.126$). Class distribution within *Proteobacteria* was generally stable over time, although the proportion of *Beta-* and *Gammaproteobacteria* in imbedded communities decreased and increased over time, respectively ($p \leq 0.035$).

3.2.2. Fungal communities

Fungal successional dynamics were in stark contrast to those observed in bacterial communities. Fungal richness did not show a strong temporal pattern in either community type (Fig. 5C,D). Richness of the loosely adhered fungal community ranged from 96 to 253 OTUs over the 89 d experiment, and richness of the imbedded fungal community ranged from 121 to 254 OTUs. The degree of change in fungal community composition over time differed between early-stage and established communities (Fig. 4B). The composition of early-stage communities shifted over time, as indicated by a significant condition × manipulation interaction (Table S5). This trend was present in both loosely adhered and imbedded communities.

Shifts in 3D community structure over time were characterized by decreased relative abundance of Basidiomycota ($p \leq 0.032$) and general increases in the relative abundance of unknown fungi and unassigned taxa (Fig. 2). These shifts were more pronounced in loosely adhered communities compared to imbedded communities. In contrast, the relative abundances of fungal phyla did not change significantly over time in

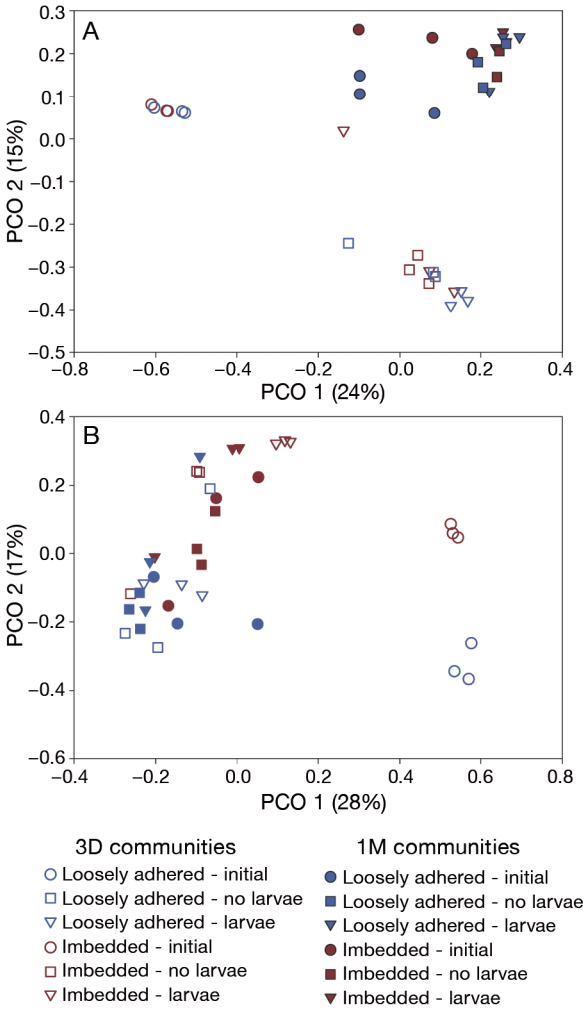

Fig. 4. Principal coordinate analysis (PCO) ordinations of (A) bacterial and (B) fungal communities across treatments. Each symbol represents a single community. 3D: 3 d; 1M: 1 mo

1M treatments. The class Leotiomycetes appeared in imbedded fungal communities following succession, but the compositional shifts were generally more subtle in both community types.

3.3. Larval effects on succession of early-stage and established communities (Prediction 3)

3.3.1. Bacterial communities

Larvae affected bacterial succession differently depending on the stage of development of the community when the larvae were added and community type. When larvae were added after 3 d of development, loosely adhered bacterial communities gained ~200 more OTUs compared to the predicted change in richness of communities without larvae over the same time period (average of 27 d, $p = 0.009$; Fig. 3E). However, adding larvae after 1 mo of community development resulted in the opposite pattern. These communities added ~200 fewer OTUs compared to the predicted richness of communities without larvae (average of 24 d; $p < 0.001$) and were less rich than the initial community (Fig. 3E). Changes in imbedded bacterial community richness over time did not differ between those with larvae and the predicted richness of communities without larvae regardless of the timing of larvae addition ($p > 0.129$; Fig. 3F). Temporal shifts in the composition of early-stage communities exposed to larvae were generally similar to those without larvae, including shifts away from *Firmicutes* and *Gammaproteobacteria* to more abundant *Bacteroidetes* and *Alpha-*, *Beta-*, and *Deltaproteobacteria* ($p < 0.003$) over time (Fig. 1). These trends were at least marginally significant in loosely adhered communities ($p \leq 0.056$). Shifts in imbedded communities were generally substantial but not significant, with the exception of the decline in *Gammaproteobacteria* over time ($p = 0.001$). 3D communities exposed to larvae also acquired the phyla *Planctomycetes* and *Armatimonadetes*. Temporal shifts in composition of established communities exposed to larvae were generally not significant. Relative abundance of *Proteobacteria* and *Firmicutes* did not change significantly over time ($p \geq 0.147$), and class distribution within *Proteobacteria* remained constant with the exception of an increase in the relative abundance of *Deltaproteobacteria* over time in imbedded communities ($p = 0.019$). Relative abundance of *Bacteroidetes* increased over time in loosely adhered ($p = 0.016$) but not imbedded communities.

3.3.2. Fungal communities

The response of fungal richness to larvae was less pronounced than that of bacterial communities. When added to early-stage communities, larvae did not affect changes in fungal richness in either community type ($p > 0.689$; Fig. 5E,F). Established loosely adhered communities exposed to larvae gained fewer OTUs over 24 d than those without larvae did over 21 d ($p = 0.027$; Fig. 5E). There was no difference in the change in richness of established imbedded communities exposed to larvae and those without larvae ($p = 0.115$; Fig. 5F).

Fungal community composition shifts over time were similar with and without larvae. When exposed to larvae, 3D fungal communities changed more drastically over time compared to 1M communities (Fig.

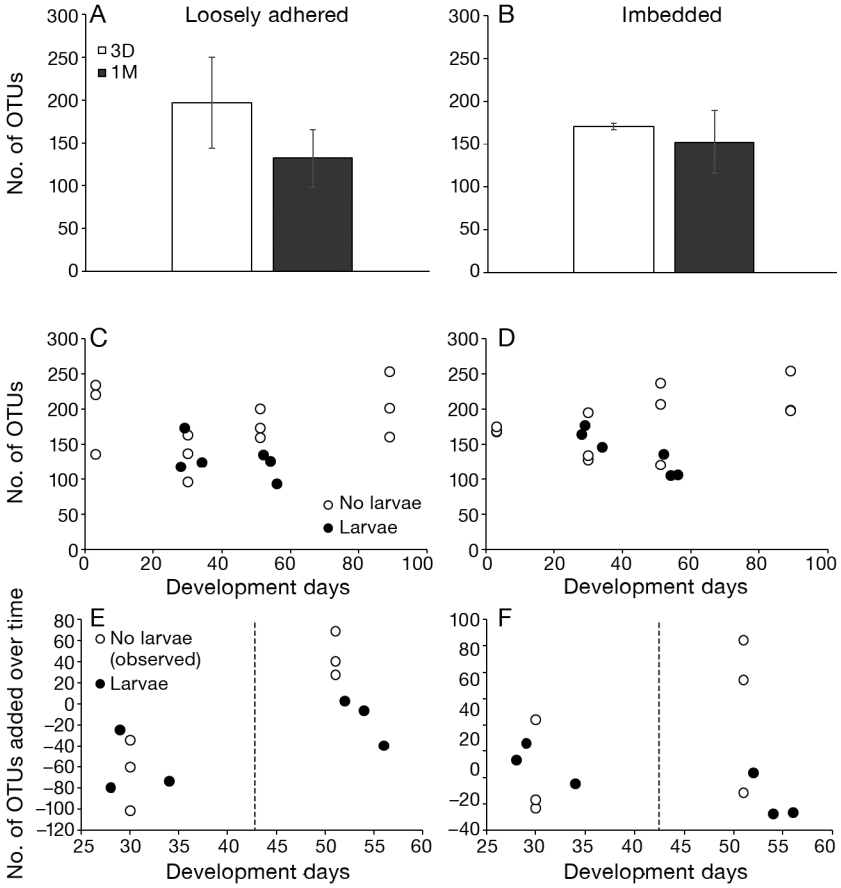

Fig. 5. Assessment of predicted changes in (A,C,E) loosely adhered and (B,D,F) imbedded fungal richness. Bars in (A) and (B) compare mean (±SD) richness of 3 d (3D) and 1 mo (1M) initial communities (Prediction 1; see Section 1 for details of predictions). (C) and (D) describe changes in richness over time (Prediction 2). (E) and (F) compare changes in richness over time in communities exposed to larvae and communities without larvae sampled after roughly the same time period (Prediction 3). Symbols to the left of vertical lines denote changes in richness in early-stage communities (e.g. 3D/larvae – 3D/initial) and symbols to the right denote changes in richness in established communities (e.g. 1M/larvae – 1M/initial).

4B, Table S5). We found a marginally significant ($p = 0.047$) interaction between community type and condition on community composition (Table S5), indicating that the difference in magnitude of compositional shifts between 3D initial and larvae communities and 1M initial and larvae communities was marginally greater for loosely adhered fungal communities than for imbedded fungal communities.

Compositional shifts over time in loosely adhered 3D fungal communities exposed to larvae included the decline in relative abundance of Ascomycota and Basidiomycota ($p \leq 0.034$) as well as the increase in relative abundance of unknown fungi and unassigned taxa ($p \leq 0.003$). Shifts in imbedded community composition included an increase in the relative abundance of Ascomycota ($p = 0.023$) and decreases in the relative abundances of Basidiomycota and unassigned taxa ($p \leq 0.032$). Temporal shifts in composition of established fungal communities exposed to larvae were weak, with no significant changes in any of the fungal phyla ($p \geq 0.083$).

4. DISCUSSION

4.1. How do leaf-associated microbial communities change over time?

Our first 2 predictions related to how microbial communities develop over time. Based on the hypothesis that the diversity of endogenous heterotrophic communities is unimodal over succession (Fierer et al. 2010), we predicted that established communities would be more diverse than early-stage communities, and that diversity would increase non-linearly over time. We found that bacteria and fungi differed sharply in their response.

In general, bacterial community dynamics strongly supported our predictions. Bacterial communities contained more OTUs after 1 mo compared to 3 d of development, and bacterial community richness was non-linear over time. Our results are similar to other studies of bacterial communities associated with a variety of substrates in aquatic ecosystems. Das et al. (2007) observed increasing bacterial richness on sugar maple *Acer saccharum* leaves until 30 d of instream incubation, followed by a period of stability and declining richness after 90 d. Mora-Gómez et al. (2016) found increased bacterial richness and diversity over the course of black poplar *Populus nigra* leaf decomposition in a Mediterranean stream. Both studies used denaturation gradient gel electrophoresis and found far fewer OTUs (≤41) than we did. Newman et al. (2015) identified 300–1000 bacterial OTUs, a range similar to ours, associated with red maple *A. rubrum* and water oak *Quercus nigra* leaves using Illumina MiSeq. They also observed increased bacterial richness over time, but not until 128 d of incubation. The fact that bacterial communities in a stream required more time to accumulate taxa than those in our mesocosms is somewhat surprising, as the rate of

immigration and diversity of the source community are presumably greater in a natural environment. However, flow regimes can influence colonization dynamics (Ferreira & Graca 2006), and the fact that tree holes are largely stagnant may explain the differences between stream and tree hole habitats.

Overall, the patterns of bacterial community richness we found support the hump-shaped pattern of diversity over succession that Fierer et al. (2010) hypothesized for endogenous heterotrophic microbial communities. While we did not observe a strong decline in diversity hypothesized during later stages of succession, it is possible that the duration of our experiment did not extend to this point. In addition to shifts in diversity, Fierer et al. (2010) predicted specific changes in composition over succession, such as the early dominance of copiotrophs followed by the gradual increase in oligotrophs. Certainly, we saw significant differences in community composition between early-stage and established communities, including a shift from *Firmicutes* and *Gammaproteobacteria* to *Bacteroidetes*, *Alpha-*, and *Betaproteobacteria* over time. *Beta-*, *Alpha-*, and *Gammaproteobacteria* have copiotrophic characteristics (e.g. high abundance in carbon-rich soils), while *Firmicutes* are more abundant in carbon-poor environments (Fierer et al. 2007), suggesting an abundance of copiotrophs throughout our study and the loss, not gain, of an oligotrophic group over time. However, these are broad characterizations (Fierer et al. 2007), and consideration at a finer taxonomic scale is instructive. The taxa abundant in later communities, including the families *Chitinophagaceae* and *Flavobacteriaceae* (Table S2), contain members capable of degrading large or complex carbon compounds such as polysaccharides, cellulose, and chitin (McBride 2014, Rosenberg 2014), functions critical for metabolizing the refractory leaf material remaining in the later stages of decomposition. Differences between 3D/initial and 1M/initial communities also likely reflected the shift from a phyllospheric to an aquatic microbiome. For example, *Alpha-* and *Betaproteobacteria* are common aquatic taxa (Nold & Zwart 1998), while *Gammaproteobacteria* have been found to be abundant on senescent leaves before submersion (Newman et al. 2015).

In contrast, our predictions were not supported by fungal communities. The response of leaf-associated fungal diversity over community development in stream ecosystems has been mixed. Mora-Gómez et al. (2016) reported stable fungal richness and diversity on poplar leaves during decomposition. Bärlocher (1980) also found relatively stable fungal richness over 200 d of instream incubation, but his first sample was not taken until after 5 wk of incubation and therefore missed any transition from early communities. Others have described increasing fungal richness over timeframes comparable to the comparison between 3D/initial and 1M/initial communities in our study (Duarte et al. 2010, Pérez et al. 2014). Factors other than timing may make comparing our results to those of stream studies difficult. Importantly, dispersal opportunities in our mesocosms were limited to the occasional inputs of fresh stemflow and atmospheric deposition, while dispersal in natural streams is much less constrained. While natural tree holes would almost certainly experience more exogenous inputs than our laboratory surrogates, these inputs would still likely be reduced compared to streams.

Patterns of fungal diversity over time did not readily fit the endogenous heterotrophic microbe succession model of Fierer et al. (2010). In his review of aquatic hyphomycetes, Suberkropp (1992) suggested that the core members of the fungal community are rarely replaced over time. If so, the establishment of a core community with stable diversity occurred rapidly in our study (<3 d). We did observe differences in fungal community composition over succession. However, the temporal shifts observed in this and other studies (Das et al. 2007, Duarte et al. 2010, Mora-Gómez et al. 2016) may be due to the acquisition and loss of rare taxa, as suggested by Suberkropp (1992) and other early studies of fungal communities (e.g. Bärlocher 1980). Modern molecular techniques likely capture more rare taxa than did the microscopy- or culture-dependent methods used in older studies. Indeed, we found that the total relative abundance of the most abundant fungal OTUs was lower in later communities (Table S3), suggesting an increased abundance of rare taxa over succession. The temporal dynamics and function of these rare taxa during decomposition is a potentially fruitful avenue of future research (Shade & Gilbert 2015).

4.2. How does predation influence successional trajectories of early-stage and established communities?

Our third prediction was that the response of microbial communities to predation would depend on the stage of community development, specifically, that larvae would suppress diversity in early communities. We observed larvae effects only in loosely adhered communities, and these effects were oppo-

site of what we predicted. We found that larvae added early in succession increased community richness while larvae added later in succession suppressed richness. Larvae generally had stronger impacts on bacterial communities compared to fungal communities.

There are several possible reasons why larval effects were not as we predicted. When introduced early in succession, grazing by larvae may prevent the establishment of dominant taxa, thereby increasing diversity (i.e. the intermediate disturbance hypothesis). Herbivores often influence the structure of grazed communities by the selective grazing of dominant species, resulting in reduced competition among species (e.g. Lubchenco 1978, Mortensen et al. 2018). Larvae may be less effective in preventing dominance when introduced to an established community. Larval traits may also have played a role in our results. *Aedes triseriatus* larvae have a wide range of feeding behaviors (Walker & Merritt 1991), which include filter feeding as well as browsing, and these behaviors change as the larvae develop; early instars rely more heavily on filter feeding (Juliano & Reminger 1992). Filter feeding could reduce the impact of larvae on leaf-associated microbes. Studies have shown significant reductions in leaf-associated bacterial abundance and production in the presence of *A. triseriatus* larvae in mesocosms similar to the ones we used (Kaufman et al. 1999, 2001), but the patterns we observed may have resulted from an interaction between the stage of community development and larval feeding mode. The weaker response of fungi to larvae may also be due to larval feeding behavior. The effects of *A. triseriatus* larvae on fungal abundance are variable (Kaufman et al. 1999, 2001, 2008), thought to be due in part to the inability of larvae to access fungal hyphae imbedded in the leaf tissue. The lack of larval impact on imbedded fungal communities in our experiment supports this hypothesis. Finally, larvae may have had indirect impacts on microbial communities other than predation. Consumer nutrient recycling significantly contributes to bottom-up community regulators such as resource availability in aquatic ecosystems (Vanni 2002), and it may have relieved nutrient limitation in early-stage communities, leading to increased richness over time.

We have focused on the comparison of temporal diversity patterns of 3D and 1M communities with and without larvae to determine how larvae influence successional outcomes of communities in different stages of establishment. A direct comparison of the end point communities with and without larvae (i.e. 3D/no larvae treatments compared to 3D/larvae treatments) is difficult because these endpoints were sampled at different times. However, no larvae and larvae communities within the same conditioning treatment were more similar to each other than either community was to the initial community, suggesting that larvae weakly affected community composition following succession. Predation by meiofauna can affect heterotrophic microbial communities, with effect type and strength depending on species-specific traits (Bell et al. 2010, Glücksman et al. 2010) The reported effects of macroinvertebrates on leaf-associated microbial community diversity and composition in other aquatic environments are mixed. Several studies have shown relatively weak effects of macroinvertebrates on microbial community diversity (Suberkropp & Wallace 1992, Ferreira & Graca 2006, Chung & Suberkropp 2008, Mora-Gómez et al. 2016), while others have shown positive (Rossi 1985, Sabetta et al. 2000) and negative (Bärlocher 1980, Mora-Gómez et al. 2016) effects on diversity and significant shifts in composition (Moghadam & Zimmer 2014, 2016, Móra-Gomez et al. 2016). *A. triseriatus* larvae have been shown to affect bacterial and fungal community composition in natural tree holes and laboratory surrogates (Kaufman et al. 1999, 2008).

A notable difference in endpoint communities with and without larvae in our study was the relatively high abundance of *Flavobacterium* in communities exposed to larvae (Table S2). Studies of natural tree holes have shown depressed relative abundance of *Flavobacteriaceae* in the water column in the presence of larvae, with little change in abundance within leaf-associated communities (Kaufman et al. 2008, Xu et al. 2008). Our results support differential larval effects on waterborne and leaf-associated *Flavobacteriaceae*, which should be considered when evaluating the potential of this group as an engineered biological control agent (Chen et al. 2014).

4.3. Bacterial versus fungal dynamics

The finding that fungal and bacterial diversity did not have the same successional pattern suggests that although both communities are endogenous heterotrophic communities (i.e. fueled by substrate carbon; Fierer et al. 2010), they respond to different successional drivers. Similar differences in fungal and bacterial successional trajectories have been observed in other systems (Brown & Jumpponen 2014, Poosakkannu et al. 2017), suggesting that mechanisms driving these differences may be widespread. The

assembly of microbial communities is determined in part by environmental filters (Kraft et al. 2015) which can constrain phylogenetic diversity, as closely related taxa are likely to filter similarly (Webb et al. 2002). Brown & Jumpponen (2014, 2015) found that fungal richness, diversity, and phylogenetic diversity remained stable over early primary succession in deglaciated soils, while bacterial communities were much more dynamic and suggested that this disparity occurred because niche space for fungi may be limited in these soils. A similar dynamic may be occurring on the leaf substrates. The ability to produce exoenzymes and to infiltrate the leaf matrix (Suberkropp & Klug 1980, Suberkropp et al. 1983) allows fungi to access substrate carbon unavailable to most bacteria, creating a relatively unique but narrow niche, while greater metabolic diversity allows bacteria to fill more niches. We suggest that water-filled tree holes are ideal habitats for testing this theory, as they are largely heterotrophic ecosystems and are easily replicated in the field or laboratory. Understanding how changes in bacterial and fungal communities over time are related to organic matter decomposition in these systems will further ecological theory and may contribute to mosquito control efforts.

Acknowledgements. We thank Michael Kaufman, Jennifer Kirk, Alexander Ruhs, and Andrew Van Alst for assistance with the experiment. We also thank Ashley Shade, Jackson Sorenson, Aaron Garoutte, and Pat Bills for their assistance with the bioinformatics processing. Sequencing and computing resources were provided in part by the Michigan State University Institute for Cyber-Enabled Research and the genomics facility with the Michigan State University's Research Technology Support Facility. This research was supported by NIH grant R37AI21884.

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Editorial responsibility: Jennifer Martiny, Irvine, California, USA

Submitted: December 7, 2018; Accepted: July 9, 2019
Proofs received from author(s): October 2, 2019