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Early bacterial and fungal colonization of leaf litter in Fossil Creek, Arizona

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Abstract. Microbes are important in stream ecosystem processes and ubiquitous in stream environments, but limitations of study techniques have left most of these microbial communities poorly described. In stream ecosystems, fungal and bacterial communities play critical roles in leaf decomposition and release energy and nutrients to higher trophic levels of the food web. Our research examined microbial communities in Fossil Creek, Arizona, USA, to elucidate effects of litter quality and abiotic habitat characteristics on early microbial colonizers of leaves. High- and low-quality leaf litter was placed in the creek at 5 study sites with heterogeneous environmental conditions (including differing stream morphology, water flow, water chemistry, and travertine deposition). Microbial assemblages that colonized the decomposing leaves were characterized using terminal-restriction fragment length polymorphism analysis and clone library comparisons. Our study revealed differences in microbial community structure along environmental gradients and, to some extent, between high- and low-quality litter in Fossil Creek. Leaf decomposition rates were strongly influenced by both litter quality and abiotic site characteristics, but microbial communities were more strongly influenced by site than by litter quality. Bacterial and fungal communities differed with incubation times: bacterial diversity increased between 2-d and 8- to 9-d incubations, whereas fungal diversity decreased. Fungal community diversity was negatively correlated with decomposition rates after incubation in the creek for 2 d when the community still included nonaquatic fungi, but this relationship did not exist after longer incubation. Bacterial community diversity was not related to litter quality or decomposition rates.

Key words: microbial community, leaf litter, bacteria, fungi, molecular methods, travertine stream, calcium carbonate.

Headwaters and low-order streams are typically heterotrophic systems that rely more on detrital inputs than on primary production (Cummins 1974, Vannote et al. 1980). In temperate deciduous forests, leaves are the major source of riparian inputs (Lamberti and Gregory 1996, Abelho and Graça 1998), and leaf litter exclusion studies show that these inputs structure stream food webs and affect ecosystem productivity (Wallace et al. 1997, Johnson et al. 2003). Leaf litter decomposition rates, which indicate how rapidly leaf energy and nutrients enter the stream food web, are influenced by leaf and stream characteristics, including N and lignin concentrations in leaves, water chemistry, stream velocity, and macroinvertebrate shredder communities (reviewed in Webster and Benfield 1986, Suberkropp 1998).

In stream ecosystems, fungal and bacterial communities play important roles in the breakdown of leaves, especially during initial stages of decomposition. Bacterial and fungal enzymes break down recalcitrant leaf compounds and release nutrients to higher trophic levels. Microbial conditioning makes leaves palatable to macroinvertebrates (Arsuffi and Suberkropp 1985, Graça et al. 2001), which probably obtain more nutrients and energy from microbial colonizers than from the leaf itself (Kaushik and Hynes 1971, Cummins 1974, Graça 2001). Some macroinvertebrates feed selectively on particular fungal species or microbial assemblages on leaves (Petersen and Cummins 1974, Suberkropp et al. 1983, Suberkropp and Arsuffi 1984, Arsuffi and Suberkropp 1986, 1989, Butler and Suberkropp 1986), a behavior that suggests qualitative differences among microbial communities.

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The roles of macroinvertebrates in leaf litter decomposition have been studied extensively, and macroinvertebrate communities are well characterized (see references in Graça 2001), but considerably less is known about microbial communities involved in leaf decomposition (reviewed in Suberkropp 1998). Leaves are colonized by aquatic fungi and bacteria within 10 d of entering stream ecosystems (Paul et al. 1977), yet little is known about these early microbial communities.

Molecular methods complement techniques based on culturing or conidial morphology to characterize bacterial and fungal communities and provide additional insight into microbial community structure. Terminal-restriction fragment length polymorphism (T-RFLP) generates community fingerprints based on deoxyribonucleic acid (DNA) sequence differences (Liu et al. 1997). Despite intrinsic biases, T-RFLP provides reasonable estimates of microbial community structure and diversity (Muyzer et al. 1993, Amann et al. 1996, Liu et al. 1997, Clement et al. 1998, Marsh 1999, Osborn et al. 2000, Dahllöf 2002, Forney et al. 2004) and is generally accepted for characterizing aquatic bacterial and fungal communities (Dorigo et al. 2005, Nikolcheva and Bärlocher 2005, Mitchell and Zuccaro 2006).

Recent molecular studies have characterized and compared aquatic microbial communities along environmental gradients (Battin et al. 2001, Sekiguchi et al. 2002, Araya et al. 2003, Brümmer et al. 2003, Feris et al. 2003, Williams and Fulthorpe 2003). These qualitative and semiquantitative studies reached limited conclusions, but molecular tools have revealed great phylotype diversity within and variability among microbial communities. Molecular studies of aquatic fungal communities on decomposing leaves suggest that conidia morphotyping underestimates diversity (Nikolcheva and Bärlocher 2004, Nikolcheva et al. 2005).

Composition of microbial communities in streams has been related to abiotic habitat characteristics (Battin et al. 2001, Williams and Fulthorpe 2003) and to substrate type and quality (Nikolcheva et al. 2003, Fazi et al. 2005), yet no study has systematically determined the relative importance of habitat vs substrate quality for microbes in streams. Our study used T-RFLP to determine if abiotic site characteristics or organic substrates were more important for structuring early communities colonizing leaves. Decomposition rates and microbial communities were compared on high- and low-quality leaf litter (rapidly and slowly decomposing leaves, respectively) at 5 sites in Fossil Creek, Arizona, USA, that differed in flow, stream morphology, water chemistry and temperature, and travertine deposition. From Fossil Springs to the confluence with the Verde River, concentrations of dissolved inorganic nutrients and Ca decrease and pH increases (Marks et al. 2005). Calcium carbonate precipitates as discrete travertine terraces in upstream reaches and as continuous layers or crusts coating the streambed in downstream reaches. Leaf decomposition rates tend to be correlated with litter quality, but macroinvertebrate shredder communities in Fossil Creek are more strongly structured by abiotic variables than by litter quality (LeRoy and Marks 2006). Abiotic stream characteristics were expected to have a stronger influence than substrate quality on microbial community structure because of the heterogeneity of environmental conditions along the stream and based on the responses of macroinvertebrate assemblages to these habitat variables.

The relationship between biodiversity and ecosystem function continues to be explored, and more studies on ecosystem processes in aquatic ecosystems, especially of decomposition and microbial communities, are needed (reviewed in Schläpfer and Schmid 1999, Giller and O'Donovan 2002, Giller et al. 2004). In general, greater diversity has been linked with increased ecosystem process rates. We compared microbial communities at 2 incubation times to test for temporal changes in diversity. Within a particular habitat, we predicted that microbial diversity would be positively correlated with litter decomposition rates.

Methods

Study sites

Fossil Creek is a perennial headwater stream at the southern edge of the Colorado Plateau in central Arizona. The creek has a discharge of 1218 L/s and flows ~22.5 km from Fossil Springs to the confluence with the Verde River. The springs have high concentrations of dissolved CO_2 and Ca. As CO_2 outgases, the stream becomes supersaturated with calcite, which precipitates as travertine. Travertine terraces form a distinct upstream reach, whereas continuous layers or crusts of travertine precipitate along downstream reaches.

A hydropower dam and flumes were built in 1909 to divert the water from Fossil Creek to 2 hydroelectric plants. Downstream of the Irving Power Plant, ~150 L/s of creek water was returned to the stream forming a 1-km travertine terrace reach (Marks et al. 2005). Our study was conducted in spring 2004 before the hydropower dam was decommissioned and full flows were restored to the creek in June 2005.

Five study sites were chosen along the environmental gradients in Fossil Creek and were numbered from

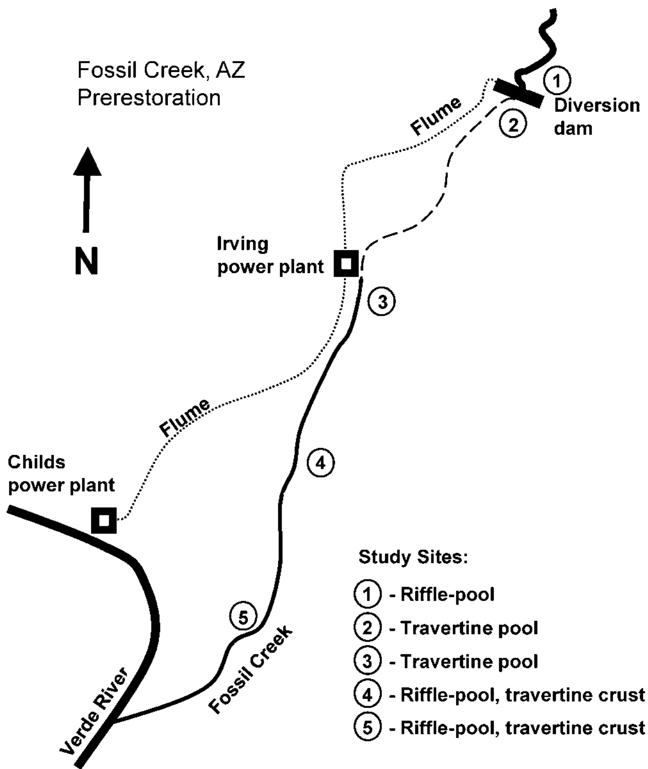

FIG. 1. Fossil Creek, Arizona (AZ) in spring 2004 before the hydropower dam was decommissioned and full flows were restored to the creek (not to scale).

upstream to downstream (Fig. 1). Site 1 was ~0.25 km from the springs in the riffle–pool reach upstream of the diversion dam and travertine deposition and had a cobble and gravel streambed. Site 2 was in the dewatered reach below the diversion dam (~0.5 km from the springs) where <6 L/s seeped under the dam and formed a diminutive travertine terrace reach with small terraces and pools. Site 3 was below Irving Power Plant, ~7 km below the springs, in the travertine terrace and pool reach. Both travertine terrace reaches had solid travertine dams and loose, gravel-sized travertine in the pools. Site 4 was in the riffle–pool reach ~12 km below the springs, and site 5 was in the riffle–pool reach ~16 km below the springs. In these downstream reaches, travertine precipitated in continuous layers armoring the creek channel, with small amounts of loose gravel and finer sediments in the streambed. The 3 upstream sites were warmer than the 2 downstream sites (mean water temperatures: 22.0°C, 20.0°C, 21.7°C, 18.8°C, and 16.8°C for sites 1 through 5, respectively) and contained greater concentrations of dissolved Ca, PO_4^{3-}, and N (Marks et al. 2005). Proceeding downstream, pH increased from neutral at site 1 to between pH 8 and 9 at site 5 (Malusa et al. 2003, Marks et al. 2005).

Leaf litter decomposition

Leaf species used in litter bags were Arizona alder (*Alnus oblongifolia*), a species with leaves that decompose rapidly, and Arizona sycamore (*Platanus wrightii*), a species with leaves that are recalcitrant to decomposition (LeRoy and Marks 2006). Rapid and recalcitrant decomposing leaves were used to test how microbial communities respond to litter quality (high and low, respectively). Alder, sycamore, and cottonwoods (*Populus* spp.) are the dominant riparian plants in the Fossil Creek ecosystem. Leaves were collected on tarps during autumn 2002 and allowed to air dry. Approximately 4 g of dry alder leaves and 3 g of dry sycamore leaves were sewn into litter bags made of Vexar netting (DuPont, Washington, DC) with 6-mm mesh size. Exact masses were recorded. Litter bags were attached to rebar and placed in Fossil Creek at study sites 1, 2, and 3 on 12 March 2004 and at study sites 4 and 5 on 13 March 2004. Leaf litter, especially recalcitrant litter including sycamore leaves, was present in the stream on these spring dates. For each leaf species, 8 time-0 litter bags (not placed in the creek) were used to estimate mass loss caused by field transport.

On harvest dates, 8 litter bags of each species were collected at each site. Litter bags at sites 1, 2, and 3 were removed after being submerged for 9, 37, 68, and 89 d. Litter bags at sites 4 and 5 were removed from the creek after being submerged for 8, 36, 67, and 88 d. Leaves were rinsed with distilled water to remove silt, debris, and macroinvertebrates; dried at 70°C; and weighed. Each sample was ground to ~425 μm using a Wiley Mill and a subsample was weighed, combusted at 500°C for 30 min, and reweighed to determine ash-free dry mass (AFDM).

Microbial community analyses

DNA extraction.—On each harvest date, 3 litter bags of each species were collected at each site. Litter bags at sites 1, 2, and 3 were removed after being submerged for 2 d (time 1) and 8 d (time 2). Litter bags at sites 4 and 5 were removed after being submerged for 2 d (time 1) and 9 d (time 2). A 5-mm-diameter cork borer was used to collect ~50 leaf punches from each litter bag. Punches were stored on glycerol at −20°C for community DNA extraction.

Subsets of punches from each litter bag were processed separately with the FastDNA Spin Kit for Soil (Qbiogene, Solon, Ohio) and the UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, California). Manufacturers' protocols were modified to obtain maximum yields of genomic DNA. Bead tubes containing leaf punches and lysing solutions were incubated at 65°C for 10 min, then homogenized for

30 s on a Mini-Beadbeater (Biospec Products, Bartlesville, Oklahoma), and filters were washed additional times before DNA elution. For each litter bag, extracted genomic DNA obtained with the 2 kits was visualized by agarose gel electrophoresis to verify quality and quantity and then pooled before polymerase chain reaction (PCR).

T-RFLP.—Microbial communities were characterized via T-RFLP analysis based on 16S ribosomal DNA (rDNA) sequences for bacteria and internal transcribed spacer (ITS) ribosomal region sequences for fungi. Protocols for PCR and restriction endonuclease digests were optimized in a series of preliminary experiments (data not shown).

Bacterial 16S rDNA was amplified from community DNA using primers 8F-FAM (5′-/56-FAM/AGAGTTTGATCCTGGCTCAG-3′) and 907R1 (5′-CCGTCAATTCCTTTGAGTTT-3′). The forward primer, 8F-FAM, was labeled with 5(6)-carboxyfluoresceine. Each reaction included 1× PCR buffer (Qiagen, Valencia, California), 200 μM of each dNTP (Qiagen), 0.5 μM of each primer (Integrated DNA Technologies, Inc., Coralville, Iowa), 0.4 mg/mL bovine serum albumin, 2.5 U Taq DNA polymerase (Qiagen), 2 μL community DNA, and molecular-grade water added to a final volume of 50 μL. Molecular-grade water replaced DNA template in negative controls. The thermal cycler protocol included initial denaturation at 94°C for 3 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s; and final elongation at 72°C for 5 min. Amplicons were visualized by agarose gel electrophoresis.

Fungal ITS regions were amplified from community DNA using primers ITS1-F-FAM (5′-/56-FAM/CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) that target the partial 18S rDNA sequence, complete ITS 1 sequence, complete 5.8S rDNA sequence, complete ITS 2 sequence, and partial 28S rDNA sequence (White et al. 1990). The forward primer, ITS1F-FAM, was labeled with 5(6)-carboxyfluoresceine. Each 50-μL reaction had the same reagents as described for bacteria except that 0.2 μM of each primer was used. Molecular-grade water replaced DNA template in negative controls. The thermal cycler protocol included initial denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 60 s, and elongation at 72°C for 60 s; and final elongation at 72°C for 10 min. Amplicons were visualized by agarose gel electrophoresis.

Bacterial 16s rDNA amplicons were digested using restriction endonucleases *Hae*III and *Msp*I. Fungal ITS amplicon digestions used endonucleases *Hae*III and *Rsa*I. Each digest included 1× RE buffer (Promega, Madison, Wisconsin), 0.1 mg/mL bovine serum albumin, 0.25 U/μL of each endonuclease (Promega), 3 μL PCR product, and molecular-grade water added to a final volume of 20 μL. Amplicons were digested at 37°C for 6 h, then visualized by agarose gel electrophoresis.

Digested DNA was precipitated and stored at −20°C. DNA was resuspended in 10.8 μL Hi-Di formamide (Applied Biosystems, Foster City, California) and 0.2 μL X-Rhodamine MapMarker 1000 (BioVentures, Inc., Murfreesboro, Tennessee) and was denatured at 96°C for 5 min before capillary electrophoresis. An Applied Biosystems 3730 DNA sequencer and GeneMapper software (version 4.0; Applied Biosystems) were used for fragment length analysis at Northern Arizona University's Environmental Genetics and Genomics (EnGGen) Laboratory.

To control for PCR bias and sequencer run variation, duplicate PCRs were run for each community DNA sample, each PCR product was digested separately, and each digest was run separately on the sequencer. Terminal restriction fragments (TRFs) that appeared in duplicate T-RFLP profiles (within 0.5 basepairs [bp] in length) and were $\geq$0.5% of the total fluorescent intensity were included in community analyses.

Three community profiles were generated for each treatment, resulting in 60 bacterial community profiles and 60 fungal community profiles. Richness (S) was determined for each community profile (where $S =$ total number of TRFs). Relative abundance (P_i) was calculated for each TRF within each community profile based on fluorescent intensity (where $P_i =$ the proportion of fluorescent intensity in the total attributable to the ith TRF). The Shannon diversity index (H), was calculated for each community profile: $H = -\Sigma P_i \ln(P_i)$, where P_i is the relative abundance of each TRF as described above. Evenness (E) was calculated for each profile: $E = H/H_{max}$ and $H_{max} = \ln(S)$, where H is the diversity of TRFs as described above, and H_{max} is the maximum value of H if all TRFs had the same fluorescent intensity.

Clone libraries.—Clone libraries were generated to assign probable identities to TRFs in microbial community profiles. Four clone libraries were produced: one each for bacteria and fungi at travertine terrace sites (sites 2 and 3) and one each for bacteria and fungi at riffle–pool sites (sites 1, 4, and 5). Bacterial 16S rDNA and fungal ITS regions were amplified from the same community DNA extractions that were used to generate T-RFLP community profiles. The PCR protocols for bacterial and fungal clone libraries were the same as previously described for T-RFLP, except that neither forward primer had the 5(6)-carboxyfluo-

resceine label. PCR products were pooled for each clone library and cloned using Invitrogen's TOPO TA Cloning Kit for Sequencing with pCR4-TOPO vector and One Shot TOP10 Chemically Competent *Escherichia coli*.

Restriction fragment length polymorphism (RFLP) band patterns identified unique clones for sequencing and T-RFLP analysis. Cloned PCR products were amplified directly from the transformant cultures using primers specific to the pCR4-TOPO plasmid, T3 (5′-ATTAACCCTCACTAAAGGGA-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′), which flank the ligated PCR product in the plasmid. Reactions included 1× PCR buffer (Qiagen), 200 μM of each dNTP (Qiagen), 0.25 μM of each primer (Integrated DNA Technologies, Inc.), 0.4 mg/mL bovine serum albumin, 2.5 U Taq DNA polymerase (Qiagen), 2 μL transformant culture, and molecular-grade water added to a final volume of 50 μL. Molecular-grade water replaced DNA template in negative controls. The thermal cycler protocol included initial denaturation at 94°C for 10 min; 40 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 60 s, and elongation at 72°C for 60 s; and final elongation at 72°C for 10 min. Amplicons were visualized by agarose gel electrophoresis. Bacterial and fungal clone amplicons were digested with the same restriction digest protocol described above for T-RFLP. Digested clone amplicons were visualized by agarose gel electrophoresis.

A BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used for sequencing reactions. Bacterial clone amplicons were sequenced with primers 8F, 341F, and 907R1. Fungal clone amplicons were sequenced with primers ITS1-F and ITS4. Sequencing reactions were precipitated and stored at −20°C. DNA was resuspended in 11 μL Hi-Di formamide (Applied Biosystems) and denatured at 96°C for 5 min before capillary electrophoresis using an Applied Biosystems 3730 sequencer and Sequencing Analysis software (version 5.2, patch 2; Applied Biosystems). Reverse compliments were generated using Chromas Lite (version 2.01; Technelysium Pty Ltd, Tewantin, Queensland, Australia), and forward and reverse sequences for each clone were aligned with BioEdit Sequence Alignment Editor (version 7.0.5.3; Ibis Biosciences, Carlsbad, California) and ClustalW (European Bioinformatics Institute, Cambridge, UK). The Basic Local Alignment Search Tool (BLAST) online database (National Center for Biotechnology Information, Bethesda, Maryland) was used to compare edited clone sequences with the "nr" nucleotide sequence database. Probable identities were assigned to bacterial clones with BLAST matches >580 bp and to fungal clones with BLAST matches to both ITS1 and ITS2. The first match on the list was considered the closest uncultured relative, and the first match to a known, cultured species was considered the closest relative.

T-RFLP analysis on individual clones was similar to community T-RFLP. Bacterial 16S rDNA and fungal ITS regions were amplified directly from transformant cultures. PCR protocols and restriction digestion procedures were as described above for community T-RFLP, except that the fungal PCR was run for 30 cycles. Amplicons were cleaned using UltraClean-htp 96 Well PCR Clean-up Kit (MoBio Laboratories, Inc.). Digested amplicons were prepared for fragment analysis as described above. One PCR and digest was run for each clone.

Statistical analyses

Decomposition rates were calculated using SAS software (version 9.1; SAS Institute Inc., Cary, North Carolina) to regress ln(AFDM remaining) against the number of days in the creek. This software also was used to run contrasts to test for differences in decomposition rates between pairs or groups of treatments.

Three-way analysis of variance (ANOVA) was used to test for significant differences in microbial S, H, or E between incubation times, between leaf species, and among study sites. Linear regression analyses tested for correlations between leaf decomposition rates (k) and S, H, or E. Tukey's honest significant difference (HSD) tests were used to determine possible groupings of sites based on community diversity. JMP software (release 5.1.2; SAS Institute Inc.) was used for ANOVA, regression analyses, and Tukey's tests. T-RFLP community profiles were compared using multiresponse permutation procedures and nonmetric multidimensional scaling with Sorensen (Bray–Curtis) distance measure using PC-ORD (version 4.41; MjM Software, Gleneden Beach, Oregon).

Results

Leaf litter decomposition rates

At all study sites, alder leaves decomposed faster than sycamore leaves ($p < 0.01$ for each comparison; Fig. 2A, B). There was also a considerable difference in decomposition rates between upstream and downstream sites, with faster leaf decomposition at the 3 upstream sites than at the 2 downstream sites ($p < 0.01$; Fig. 2A, B).

Microbial community assemblages

Bacterial and fungal community diversity.—T-RFLP

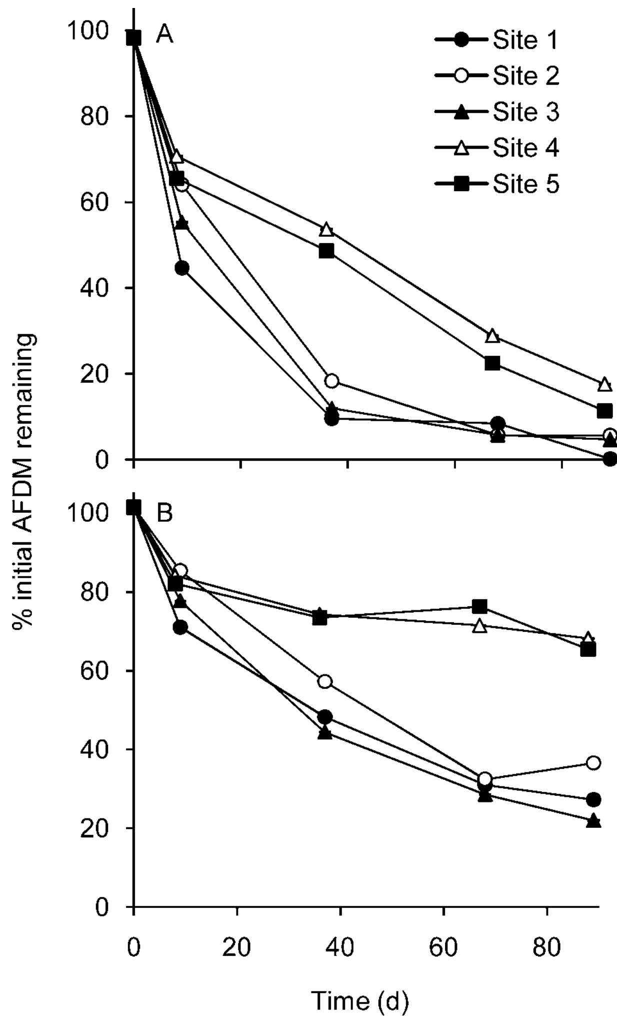

FIG. 2. Mean ($\pm$1 SE; $n = 8$) % ash-free dry mass (AFDM) of leaf litter remaining for alder (A) and sycamore (B) leaves at 5 sites along environmental gradients in Fossil Creek.

analyses revealed 120 unique bacterial phylotypes and 228 unique fungal phylotypes from all litterbag communities. For each treatment, fungal S and H were greater than bacterial S and H, whereas E was similar between fungal and bacterial communities. Bacterial S ranged from 4 to 36 phylotypes (mean $S =$ 15 phylotypes), H ranged from 0.63 to 3.03 (mean $H =$ 1.99), and E ranged from 0.45 to 0.96 (mean $E = 0.78$). Fungal S ranged from 6 to 43 phylotypes (mean $S = 21$ phylotypes), H ranged from 0.95 to 3.25 (mean $H =$ 2.29), and E ranged from 0.41 to 0.91 (mean $E = 0.76$).

Incubation time and site strongly structured both microbial communities (Tables 1, 2). Litter type affected fungal S and H but had no significant effects on bacterial diversity (Tables 1, 2). Bacterial and fungal communities changed dramatically between incubation times (Fig. 3A, B, Tables 1, 2). However, bacterial S, H, and E increased with time (between times 1 and 2, mean S increased from 12.9 to 17.3, mean H from 1.75 to 2.24, and mean E from 0.72 to 0.83), whereas fungal S and H decreased with time (between times 1 and 2, mean S decreased from 25.5 to 16.5 and mean H from 2.53 to 2.06). Habitat characteristics also influenced community structure. In general, microbial communities were associated with travertine deposition. This pattern was most pronounced for bacteria at time 1 when communities were similar among sites 2, 3, and 4 (Fig. 3A), which all had travertine terraces or crusts. At time 1, fungal communities at sites 2 and 3 (travertine terrace reaches) differed from communities at the other sites (riffle–pool reaches) (Fig. 3B). At time 2, fungal communities at the 3 upstream sites differed from communities at the 2 downstream sites, and communities at the 2 travertine terrace sites were very similar (Fig. 3B). Fungal H and E were greater at riffle–pool sites than at travertine terrace sites (Tukey's HSD, $F = 9.20$, $p < 0.01$).

Analysis of bacterial and fungal clone libraries.—The bacterial clone library for travertine pool sites had 90 clones, of which 37 had unique RFLP patterns. The riffle–pool library had 93 clones, with 41 unique clones. Based on closest identified relatives (Table 3), the dominant bacterial groups in travertine and riffle–pool reaches were β-proteobacteria (54.4% and 53.8% of clones for travertine and riffle–pool libraries, respectively) and α-proteobacteria (20.0% and 25.8% of clones for travertine and riffle–pool libraries, respectively). δ-proteobacteria, γ-proteobacteria, *Bacteroides*, *Firmicutes*, and *Cyanobacteria* were minor groups in the travertine reaches, whereas *Bacteroides*, *Planctomycetes*, *Verrucomicrobia*, *Actinobacteria*, and *Gemmatimonadetes* were minor groups in riffle–pool reaches. For a few clones in both libraries, BLAST searches identified the closest relatives as a combination of uncultured bacteria and known diatom chloroplast sequences.

The fungal clone library for travertine pool sites had 96 clones, of which 35 had unique T-RFLP patterns. The riffle–pool library had 95 clones, of which 47 were unique. Ascomycota was the dominant fungal group with 85.4% and 86.3% of clones in the travertine and riffle–pool libraries, respectively. Chitridiomycota (8.3% and 8.4% for travertine and riffle–pool reaches, respectively) and Basidiomycota (2.1% and 5.3% for travertine and riffle–pool, respectively) were minor fungal groups for both travertine and riffle–pool reaches. The travertine library also had members of Glomeromycota and 2 clones that matched nonfungal eukaryotes. These 2 metazoan sequences poorly matched the clone sequences, with matching sequence lengths <300 bp and similarities between 90% and

TABLE 1. Results of 3-way analysis of variance testing on the effects of incubation time, leaf species, study site, and interactions on microbial community richness (*S*), diversity (*H*), and evenness (*E*). Significant effects are shown in bold ($\alpha = 0.05$).

	S				*H*				*E*			
Source	df	MS	*F*	*p*	df	MS	*F*	*p*	df	MS	*F*	*p*
Bacteria												
Time	1	286.02	5.83	**0.02**	1	3.53	80.90	**<0.01**	1	0.18	23.26	**<0.01**
Leaf	1	18.15	0.37	0.55	1	0.08	1.93	0.17	1	0.00	0.60	0.44
Site	4	154.71	0.27	0.60	4	0.47	10.72	<0.01	4	0.02	1.39	0.24
Time × leaf	1	6.02	0.12	0.73	1	0.02	0.42	0.52	1	0.01	0.69	0.41
Time × site	4	332.89	11.13	**<0.01**	4	1.73	39.53	**<0.01**	4	0.02	9.18	**<0.01**
Leaf × site	4	41.11	0.11	0.74	4	0.09	1.97	0.12	4	0.01	0.73	0.40
Time × leaf × site	4	96.56	1.63	0.21	4	0.34	7.70	**<0.01**	4	0.01	0.22	0.64
Fungi												
Time	1	1215.00	39.85	**<0.01**	1	3.36	35.54	**<0.01**	1	0.03	2.72	0.11
Leaf	1	180.27	5.91	**0.02**	1	0.49	5.23	**0.03**	1	0.01	0.44	0.51
Site	4	125.00	1.88	0.18	4	1.73	18.28	**<0.01**	4	0.10	0.03	0.87
Time × leaf	1	64.07	2.10	0.15	1	0.22	2.38	0.13	1	0.01	0.69	0.41
Time × site	4	9.75	**0.03**	0.86	4	0.22	2.37	0.07	4	0.01	0.17	0.68
Leaf × site	4	14.52	0.51	0.48	4	0.07	0.76	0.56	4	0.01	0.69	0.41
Time × leaf × site	4	45.23	5.75	0.02	4	0.32	3.40	**0.02**	4	0.02	1.99	0.16

94%. A few fungal identities matched aquatic hyphomycete species previously identified on decomposing leaves: *Alatospora acuminata*, *Anguillospora longissima*, *Tetracladium marchalianum*, and *Tricladium angulatum* (Nikolcheva et al. 2003, 2005, Nikolcheva and Bärlocher 2005; Table 4).

Probable identities of bacterial and fungal TRFs appearing in T-RFLP community profiles are shown in Tables 3 and 4. Although these tables do not provide a comprehensive list of all TRFs, some patterns emerged. Bacterial TRF 210.9 (β-proteobacteria) and TRF 135.1 (α-proteobacteria) were commonly found in community profiles and had high relative abundances. In general, bacterial TRF 135.1 increased in abundance between times 1 and 2, whereas TRF 210.9 decreased between times 1 and 2. The dominant fungal community member, TRF 123.3 (plant-associated ascomycetes), increased between times 1 and 2. Fungal TRF 86.1 (powdery mildew *Phyllactinia guttata*) was found commonly in communities at time 1, but was absent or had decreased dramatically in relative abundance by time 2. Fungal TRFs 127.3 and 128.3 (aquatic hyphomycetes *T. angulatum* and *A. acuminata*) increased in relative abundance between times 1 and 2.

TABLE 2. Results of multiresponse permutation procedures test for effects of incubation times, leaf species, and study sites on microbial community profiles. *A* = chance-corrected within-group agreement. Significant effects are in bold ($\alpha = 0.05$).

	A	*p*
Bacteria		
Time	**0.2084**	**<0.01**
Leaf	–0.0049	0.79
Site	**0.0747**	**<0.01**
Fungi		
Time	**0.0775**	**<0.01**
Leaf	0.0028	0.22
Site	**0.1929**	**<0.01**

Microbial community structure and ecosystem function

Fungal *S* at time 1 was negatively related to overall decomposition rates ($F = 6.21$, $p = 0.04$, $R^2 = 0.44$; Fig. 4A) and to the earliest decomposition rate data at 8 or 9 d ($F = 8.96$, $p = 0.02$, $R^2 = 0.53$), results that suggest a negative relationship between fungal *S* and litter quality. However, this relationship fell apart when relating fungal *S* at time 2 to either overall decomposition rates ($F = 0.12$, $p = 0.74$, $R^2 = 0.02$; Fig. 4B) or 8- or 9-d rates ($F = 0.12$, $p = 0.74$, $R^2 = 0.02$). The time 1 relationships between fungal *S* and decomposition rates were the only significant correlations between microbial community diversity and decomposition rates. Decomposition rates were not correlated with water temperatures ($F = 2.91$, $p = 0.13$, $R^2 = 0.27$).

Discussion

Decomposition rates in our study were comparable to other published rates for alder and sycamore leaves in travertine streams (Casas and Gessner 1999, LeRoy and Marks 2006, Carter and Marks 2007). As expected, alder leaves decomposed faster than sycamore leaves at all 5 sites. Leaves decomposed rapidly at the 3 upstream sites and slowly at the 2 downstream sites. As suggested by other studies, rate differences among

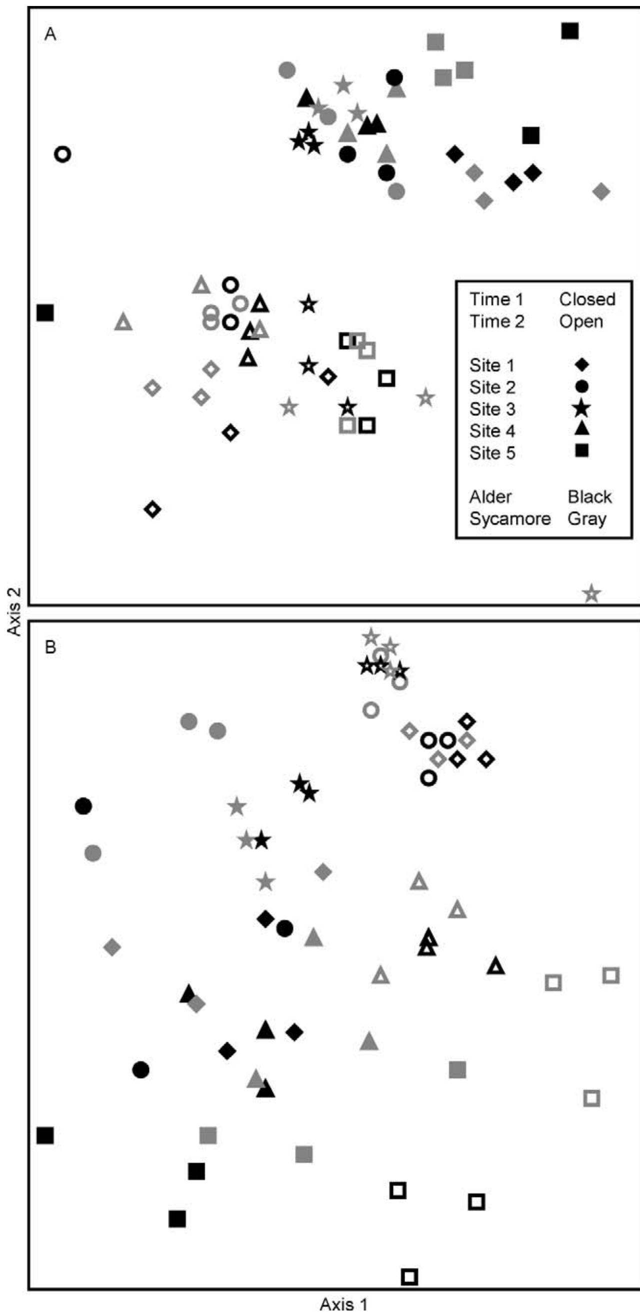

FIG. 3. Nonmetric multidimensional scaling ordination for bacterial communities (final stress for 2-dimensional solution = 12.87, final instability = 0.00028, iterations = 400) (A) and fungal communities (final stress for 3-dimensional solution = 13.70, final instability = 0.00077, iterations = 400) (B). See Fig. 1 for site description.

sites were not the result of water temperatures alone (Irons et al. 1994). Fine sediments coating litter bags and leaves at the 2 downstream sites probably contributed to decreased decomposition rates (Casas 1996). Furthermore, the 2 downstream sites had travertine deposition as continuous layers armoring the substrate.

Water chemistry probably plays a role in determining leaf decomposition rates in Fossil Creek. Microbial communities might function differently along water-chemistry gradients as pH increases and concentrations of inorganic dissolved nutrients decrease from upstream to downstream sites. Enzyme activities are altered by pH; hydrolytic enzyme activities decrease in alkaline (pH 8.2) streams (Jenkins and Suberkropp 1995). This alteration in enzyme function probably decreases the rate of cellulose breakdown (Lynd et al. 2002).

Fungal community diversity in lotic ecosystems appears to be greatly underestimated by both traditional techniques and other molecular methods. Our study documented 228 unique fungal phylotypes on leaves in Fossil Creek, whereas other recent studies reported fungal richness ranging from 10 to 84 species based on conidia morphotyping (Suberkropp 1997, Gulis and Suberkropp 2003, Pascoal and Cássio 2004, Nikolcheva and Bärlocher 2005) and 33 to 42 phylotypes via molecular techniques (Nikolcheva and Bärlocher 2005, Das et al. 2007). Environmental heterogeneity, the large number of samples analyzed, and improvements in molecular techniques probably contributed to the high diversity found in our study. Fungal richness probably was not overestimated because of amplification of nontarget eukaryotes by the ITS primer set. Clone library analyses revealed only 2 nontarget identities out of 191 total clones (and because these nontarget eukaryotes matched the clone sequence so poorly, these clones could be undescribed fungal species). Less research has targeted bacterial diversity in lotic ecosystems. Bacterial richness was greater at Fossil Creek (120 unique phylotypes) than on decomposing leaves in another stream (30 phylotypes; Das et al. 2007), but was similar to reported planktonic bacterial richness (128 phylotypes; Besemer et al. 2005).

Aquatic fungal communities on leaf litter in Fossil Creek are dominated by Ascomycota but also include Basidiomycota and Chytridiomycota, results that are consistent with both morphological and molecular studies (Nikolcheva and Bärlocher 2004). Published reports that could inform a direct comparison are lacking, but bacterial groups on decomposing leaves in Fossil Creek were similar to groups found in the water column of a large river in China, where α-proteobacteria and β-proteobacteria were dominant community members (Sekiguchi et al. 2002).

Leaf decomposition rates were strongly influenced by both litter quality and site, but microbial communities were mainly influenced by site. This result

TABLE 3. Bacterial community terminal restriction fragment (TRF) identities. Tentative identities from bacterial clone libraries matching TRFs from bacterial terminal-restriction fragment length polymorphism (T-RFLP) community profiles. BLAST = Basic Local Alignment Search Tool, bp = base pair.

			Closest uncultured relative				Closest relative		
Phylogenetic group	Clone name	TRF (bp)	GenBank description	Accession number	% similarity	Habitat	Tentative identity from BLAST	Accession number	% similarity
β-proteobacteria	BR-14	182.2	*Hydrogenophaga* sp. EMB 75	DQ413154	99	Sludge	*Hydrogenophaga defluvii*	AJ585993	97
	BT-18	187.6	Clone RVW-06	AB199573	99	Freshwater	*Sphaerotilus natans*	Z18534	98
	BR-32	191.6	Clone 04C_MJK	EF540373	94	Soil			
	BR-39	192.9	Clone ES3–22	DQ444146	98	Freshwater	*Rhodoferax ferrireducens*	CP000267	97
	BR-9	194.4	Clone T47	Z93977	96	Sludge	*Hydrogenophaga atypica*	AJ585992	94
	BT-10	194.4	Comamonadaceae clone SIB2	DQ628931	99	Glacier	*Rhodoferax ferrireducens*	CP000267	96
	BT-27	195.9	Clone T47	Z93977	98	Sludge	*Hydrogenophaga palleronii*	AF019073	95
	BT-28	209.4					*Roseateles depolymerans*	AB003624	99
	BR-16	210.9	Proteobacterium clone TAF-A31	AY038710	98	Freshwater	*Rhodoferax ferrireducens*	CP000267	97
	BR-31	210.9	Clone W-Btb7_46	DQ017919	99	Freshwater	*Aquaspirillum delicatum*	AF078756	98
	BT-1	210.9	Clone 231ds5	AY212678	98	Freshwater	*Aquamonas fontana*	AB120965	96
	BT-37	210.9	Betaproteobacterium IMCC1721	DQ664240	99	Freshwater	*Imtechium assamiensis*	AY544767	97
	BT-3	211.6	Betaproteobacterium clone MVP-55	DQ676378	99	Freshwater	*Panaciterramonas fulva*	AB245357	96
	BT-31	211.6	Betaproteobacterium LH10	DQ535024	99	Freshwater	*Rhodoferax ferrireducens*	CP000267	98
α-proteobacteria	BR-13	135.1	Clone HDBW-WB02	AB237665	95	Groundwater	*Haematobacter massiliensis*	DQ342309	97
	BT-4	141.7	Isolate BF0001B046	AM697020	98	Dust	*Devosia ginsengisoli*	AB271045	97
	BT-9	145.4	Alphaproteobacterium clone AKYG1872	AY921998	99	Soil	*Novosphingobium aromaticivorans*	CP000248	98
	BR-36	145.4	*Sphingomonas* sp. isolate UN1F1	U37345	96	Soil	*Sphingobium yanoikuyae*	AY047219	96
	BR-6	145.4					*Alterierythrobacter epoxidivorans*	DQ304436	97
	BR-26	145.4	Clone PAH-Bio-66	DQ123731	97	Soil	*Erythrobacter gaetbuli*	AY562220	97
	BT-34	182.2	Alphaproteobacterium clone DC5-50-1	AY145582	97	Marine	*Haematobacter massiliensis*	DQ342309	96
	BR-37	187.6	Clone 119b1	EF459889	99	Marine	*Nordella oligomobilis*	AF370880	94
	BR-38	216.9	Clone QHO-B5	DQ675052	99	Marine	*Agrobacterium tumefaciens*	AF508094	98
Bacteroidetes; Flavobacteria	BR-21	85.0	*Flavobacterium* sp. WB 4.3–19	AM177629	98	Freshwater	*Flavobacterium pectinovorum*	AM230490	98
	BR-29	85.0	*Flavobacterium* sp. WB 3.4.10	AM177622	99	Freshwater	*Flavobacterium pectinovorum*	AM230490	99
Actinobacteria	BR-30	216.9	Actinomycetaceae isolate SR 139	X87311	98		*Kineosporia aurantiaca*	X87110	98
Cyanobacteria	BT-33	287.9					*Phormidium autumnale*	DQ493873	96
Bacteroidetes; Sphingobacteria	BR-20	123.7	Bacteroidetes clone IRD18G07	AY947969	97	Freshwater	*Sphingoterrabacterium daejeonensis*	AB267717	85
Cyanobacteria and chloroplasts	BR-35	382.5	Cyanobacterium clone pItb-vmat-79	AB294971	94	Marine	*Coscinodiscus radiatus* chloroplast	AJ536462	90
	BR-15	385.6	Cyanobacterium clone pItb-vmat-79	AB294971	96	Marine	*Coscinodiscus radiatus* chloroplast	AJ536462	92
γ-proteobacteria	BT-26	318.1	Clone 3_22MK	EF540407	94	Soil			

TABLE 4. Fungal community terminal restriction fragment (TRF) identities. Tentative identities from fungal clone libraries matching TRFs from fungal terminal-restriction fragment length polymorphism (T-RFLP) community profiles. BLAST = Basic Local Alignment Search Tool, bp = base pair.

			Closest uncultured relative				Closest relative		
Phylogenetic group	Clone name	TRF (bp)	GenBank descriptor	Accession number	% similarity	Habitat	Tentative identity from BLAST	Accession number	% similarity
Ascomycota	FR-8	85.6					*Phyllactinia guttata*	AY870865	96
	FR-41	85.6					*Phyllactinia guttata*	AY870865	96
	FR-33	85.6					*Phyllactinia guttata*	AY870865	97
	FR-32	85.6	*Hormonema* sp. GCA 2004	AF013225	90	Plant	*Scleroconidioma sphagnicola*	AY220610	90
	FT-3	85.6					*Endoconidioma populi*	AY604526	99
	FT-34	85.6					*Endoconidioma populi*	AY604526	98
	FR-11	85.6					*Endoconidioma populi*	AY604526	98
	FR-14	86.1					*Phyllactinia guttata*	AY870865	97
	FR-13	112.9	Clone 137–74	DQ420937	99	Soil	*Phoma glomerata*	AY183371	99
	FR-17	112.9	Clone 137–74	DQ420937	99	Soil	*Phoma glomerata*	AY183371	98
	FT-6	112.9	Clone 133–25	DQ420984	99	Soil	*Phoma glomerata*	AY183371	98
	FT-1	123.3	Ascomycete sp. HKA32	DQ092513	96	Marine	*Phaeosphaeria setosa*	AF439500	93
	FR-1	123.3	Ascomycete sp. HKA32	DQ092513	97	Marine	*Phaeosphaeria setosa*	AF439500	93
	FR-26	123.3	Ascomycete sp. HKA32	DQ092513	97	Marine	*Phaeosphaeria setosa*	AF439500	93
	FR-28	123.3	Ascomycete sp. HKA32	DQ092513	97	Marine	*Phaeosphaeria setosa*	AF439500	93
	FT-4	123.3	Ascomycete sp. HKA32	DQ092513	97	Marine	*Phaeosphaeria setosa*	AF439500	93
	FT-18	127.3					*Tricladium angulatum*	AY204610	99
	FR-19	127.3					*Tricladium angulatum*	AY204609	100
	FR-34	127.3					*Tricladium angulatum*	AY204609	99
	FR-36	128.3					*Alatospora acuminata*	AY204588	98
	FR-37	132.5					*Tetracladium marchalianum*	AY204620	97
	FR-3	135.9	Clone 205–23	DQ420890	99	Soil	*Alternaria alternata*	DQ023279	99
	FR-4	135.9	Clone 205–24	DQ420890	99	Soil	*Alternaria alternata*	DQ023279	99
	FT-28	135.9	Clone 205–32	DQ420893	93	Soil	*Alternaria arborescens*	AY154706	93
	FR-42	135.9	Ascomycete isolate 1000116739	AF502786	95	Leaf litter	*Glionectria tenuis*	EF495240	94
	FT-8	143.4	Ascomycete sp. MA 4671	AJ972820	96		*Phaeosphaeria eustoma*	AJ496629	92
	FR-20	180.5					*Helicodendron luteoalbum*	EF029238	95
	FT-2	478.7	Dothioraceae sp. LM317	EF060638	98	Marine	*Aureobasidium pullulans*	AF013229	98
	FR-2	479.5					*Aureobasidium pullulans*	AF013229	98
	FT-17	575.5					*Anguillospora longissima*	AY204594	98
	FR-5	593.6					*Microdochium nivale*	EF187912	99
Basidiomycota	FR-43	135.9					*Cryptococcus randhawii*	AJ876528	84

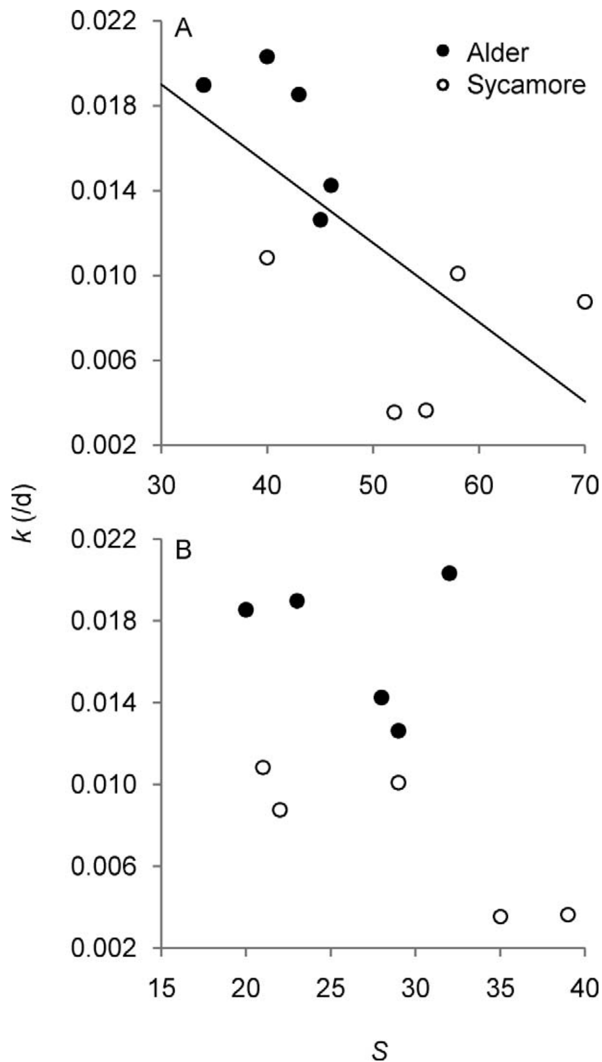

FIG. 4. Regression for leaf litter decomposition rates (k) as a function of fungal community richness (S) at time 1 (2 d) (A) and time 2 (8 or 9 d) (B).

suggests that similar communities colonize both leaf types, but have different activities on high- and low-quality litter. Other research suggests that generalist microbial communities colonize both high- and low-quality leaf litter (Das et al. 2007). These results parallel patterns of invertebrate colonization of leaf packs in which communities were strongly structured by abiotic site characteristics but showed subtle differences among litter quality (LeRoy and Marks 2006).

Time and site effects were stronger than were substrate effects, but microbial communities did differ somewhat between alder and sycamore leaves. Greater replication might be necessary to document how litter quality structures microbial communities. Significant community differences caused by interactions between site and leaf species might reflect interactions of water chemistry and substrate. For example, microbial communities responded to differences in water chemistry when colonizing low-quality substrates, but not when colonizing high-quality substrates (Fazi et al. 2005).

Bacterial and fungal communities were similar among multiple samples within sites after 2 d, whereas communities from different sites were not similar. This result suggests that aquatic microbes colonize leaves quickly. Otherwise, communities should reflect the taxa present on the leaves before submersion in the stream and would be similar among sites. Clone library analyses also suggest that the nonaquatic taxa on leaves before submersion might be replaced rapidly by aquatic species (for example, powdery mildew was common at time 1 and rare or absent at time 2). Future studies could compare communities on decomposing leaves to assemblages on leaves before submersion and to assemblages on nonorganic substrates. These analyses could generate stronger inferences about terrestrial vs aquatic microbial communities on decomposing leaves in stream ecosystems.

Microbial communities on leaves changed substantially between times 1 and 2. Bacterial diversity increased and fungal diversity decreased, a pattern also observed by Das et al. (2007). Increasing bacterial diversity after initial colonization suggests that complex communities formed as leaf nutrients became more readily available and as biofilm development created new microhabitats. Fungal T-RFLP community profiles showed that a few successful phylotypes became dominant community members after initial colonization. Other research has documented greatest fungal diversity on leaves submerged in a stream for 2 and 3 d when communities still included terrestrial members and decreasing fungal diversity over time as terrestrial fungi were replaced by aquatic hyphomycetes (Nikolcheva et al. 2005).

Contrary to predictions, microbial diversity was related to decomposition only immediately after submersion. The early correlation was driven by initial differences in fungal diversity between the 2 leaf types. After 2 d, the fungal community included nonaquatic community members and, after longer incubation, both the early relationship and the nonaquatic community members disappeared. It is possible that the composition of fungal species before submersion influences early decomposition in streams. Although the roles of endophytic fungi on leaf decomposition in streams are not clear, some endophytes were important in initial stages of terrestrial spruce needle decomposition (Müller et al. 2001), and one needle endophyte species was documented switching from an endophitic to an aquatic lifestyle (Sokolski et al. 2006).

Our results contrast with those from a lake study that showed bacterial, but not fungal, community richness was related to substrate quality (Mille-Lindblom et al. 2006).

Lower fungal richness associated with high-quality litter might indicate the dominance of a few successful fungal community members, an interpretation that was supported by some (but not all) of the T-RFLP community profiles in our study (data not shown). Alternatively, we speculate that the complex leaf chemistry of low-quality litter might provide niches for nutritional specialists with a broad range of enzymatic activities and result in increased community diversity. Differences in substrate size between the 2 leaf species might have contributed to the negative correlation between fungal richness and leaf type if larger substrates support greater fungal richness (Bärlocher and Schweizer 1983) because sycamore leaves are larger than alder leaves, and both substrate types were relatively intact after incubation. This hypothesis could be tested in future studies by constructing leaf packs with different sized leaf punches of the same litter type or the same size leaf punches of different litter types. Last, future studies that address the interactions between macroinvertebrates, microbial diversity, and leaf decomposition rates in litter bags might provide further explanation for the results of our study.

Overall, our results indicate much higher fungal S than has been previously reported and point to the potential importance of abiotic site characteristics, especially in early stages of litter decomposition in streams. Identifying and characterizing additional factors that influence this important aspect of aquatic nutrient cycling will require studies that target both individual variables and the potentially complex interactions between microbial communities, physical site characteristics, and other trophic levels.

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