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Growth of Trees under Elevated Atmospheric CO$_2$ Alters Microbial Communities Colonizing Leaf Litter in a Temperate Woodland Stream

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Abstract

Elevated atmospheric CO$_2$ can cause increased carbon fixation and altered foliar chemical composition in a variety of plants, which has the potential to impact forested headwater streams because these are detritus-based ecosystems that rely on leaf litter as their primary source of organic carbon. Fungi and bacteria play a key role in the entry of terrestrial carbon to aquatic food webs, as they decompose leaf litter and serve as a source of nutrition for invertebrate consumers. This study tested the hypothesis that changes in leaf chemistry caused by elevated atmospheric CO$_2$ would result in changes in the size and composition of microbial communities colonizing leaves in a woodland stream.

Three tree species, *Populus tremuloides*, *Salix alba*, and *Acer saccharum*, were grown under ambient (360 ppm) or elevated (720 ppm) CO$_2$ and their leaves were incubated in a woodland stream. Elevated CO$_2$ treatment resulted in significant increases in phenolic and tannin content and C:N ratios of leaves. Microbial effects, which occurred only for *P. tremuloides* leaves, included decreased fungal biomass and decreased bacterial counts. Analysis of fungal and bacterial communities on *P. tremuloides* leaves via T-RFLP and clone library sequencing revealed that fungal community composition was mostly unchanged by the elevated CO$_2$ treatment, whereas bacterial communities showed a significant shift in composition and a significant increase in diversity. Specific changes in bacterial communities included increased numbers of alphaproteobacterial and CFB-group sequences and decreased numbers of betaproteobacterial and firmicute sequences, as well as a pronounced decrease in overall Gram positive bacterial sequences.
Introduction

The concentration of atmospheric CO$_2$ has been increasing for the last 150 years, from 270 ppm prior to the industrial revolution (49) to the current level of approximately 388 ppm (http://www.mlo.noaa.gov), and is projected to exceed 700 ppm by the end of the century (57). This ongoing increase in atmospheric CO$_2$ is believed to be due to the extensive use of fossil fuels and changes in land use patterns (5). Elevated atmospheric CO$_2$ has global climate implications due to its role in the greenhouse effect (39) and it has also been shown to have direct biological effects. Specifically, elevated CO$_2$ can increase the carboxylation efficiency of ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco) (13) resulting in increased carbon fixation by C3 plants (49). This increased carbon fixation can result in increased above and below-ground plant biomass (21, 47, 63, 72) as well as altered foliar chemical composition (31, 46, 58, 70).

Elevated atmospheric CO$_2$ is unlikely to have direct impacts on forested headwater streams, as these are primarily heterotrophic systems (2) in which CO$_2$ is typically supersaturated (41). However, changes in leaf chemistry may have an impact, as forested headwater streams are detritus-based ecosystems that derive up to 99% of their carbon inputs from terrestrial organic matter (71), which is mainly leaf litter (29).

Microbes play a key role in the entry of this allochthonous organic material into stream food webs. Fungi and bacteria colonize leaf litter after its deposition in a stream and begin decomposition of the leaf material (34). The resulting growth of microbial assemblages associated with leaf litter provides a critical food resource for detritus-feeding invertebrate consumers (6, 18, 23, 44), which through their feeding activities help facilitate the further transformation and breakdown of plant litter and the flow of carbon.
and nutrients to higher trophic-level organisms, including fish. Prior research has demonstrated that aquatic invertebrates show a clear preference to eat leaves that have been extensively colonized or “conditioned” by microbes (4, 18, 65). This is likely due to the fact that microbial colonization significantly increases the nutrient content of detritus, as microbes can incorporate soluble nutrients from stream water (e.g. nitrogen) into microbial biomass (64, 66). In addition, microbes convert indigestible leaf components (e.g. lignin and cellulose) to microbial biomass, which invertebrates can digest more efficiently (6). Therefore, fungi and bacteria are significant contributors to the transfer of carbon and nutrients from terrestrial to aquatic ecosystems.

Microbial decomposition of leaves in streams is influenced by the chemical composition of the leaf material. This has been illustrated by a number of studies comparing decomposition of leaves from different tree species (for review see (62)). These studies have demonstrated that leaves from species such as oaks and conifers that are relatively high in polyphenolic compounds, including lignin and tannins, tend to decompose more slowly than leaves from species with lower concentrations of these compounds, such as alder (62). Leaf carbon to nitrogen ratio (C:N) also impacts decomposition rates; leaf litter with a high C:N tends to decompose more slowly than litter with a low C:N (62). These trends are relevant to atmospheric CO$_2$ concentrations because elevated atmospheric CO$_2$ has been shown to increase the concentrations of phenolic compounds (lignin and tannins) as well as the C:N of leaves of C3 plants (31, 46, 58, 70). Therefore, it is reasonable to hypothesize that growth of trees under elevated CO$_2$ could have negative impacts on microbial colonization and decomposition of leaves. Rier et al (58) tested this hypothesis with one tree species, *Populus tremuloides* (quaking
aspen), and found that leaves produced under elevated CO$_2$ decomposed more slowly in streams and supported less fungal and bacterial biomass than leaves produced under ambient conditions (58).

In addition to impacting microbial community size, it is reasonable to hypothesize that changes in leaf chemistry caused by growth of trees under elevated CO$_2$ could impact microbial community composition. Several studies have demonstrated that the composition of microbial communities colonizing leaves in streams can differ based on tree species (36, 45). No study we are aware of has examined the effects of tree growth under elevated atmospheric CO$_2$ on the composition of microbial communities colonizing leaf litter in streams; however, such changes in microbial community composition could be highly relevant to stream food webs. For example, different groups of fungi and bacteria differ in their ability to degrade various components of leaf litter (1, 67), so the species composition of microbial communities could potentially impact rates of decomposition and production of microbial biomass (26). This in turn could impact the transfer of carbon and energy to higher trophic level organisms. In addition, different groups of fungi and bacteria differ in chemical composition (e.g. (32)(9)) and thus they may differ in their nutritional value to aquatic invertebrates.

In the current study we tested the hypothesis that changes in leaf chemistry caused by elevated CO$_2$ would result in changes in the biomass and composition of detrital microbial communities by growing three tree species under ambient or elevated CO$_2$, collecting leaves after abscission, incubating the leaves in a woodland stream, and determining the biomass and composition of the microbial communities colonizing the leaves.
Materials and Methods

Tree Growth Under Elevated CO\(_2\)

*Populus tremuloides* (common name quaking aspen, hereafter referred to as Aspen); *Salix alba* (common name white willow, hereafter referred to as Willow); and *Acer saccharum* (common name sugar maple, hereafter referred to as Maple) were grown at the University of Michigan Biological Station (UMBS) in northern Michigan (45°34'N, 84°40'W) beginning in 2000. These tree species were chosen because they were the dominant riparian tree species at the study site, although Willow is not native to northern Michigan. Six-year-old Aspen clones, two-year-old Willow clones, and two-year-old Maple siblings (collected under one parent tree) were grown outdoors in open bottom root boxes that contained a mixture of 80% native Kalkaska rubicon sand and 20% topsoil. This mixture generated nutrient levels comparable to soils in this region (73). Each tree was placed in its own open-bottom root box, and sets of three trees (one from each species) were enclosed in 10 foot diameter x 6 foot high cylindrical clear plastic open-top chambers. Air was circulated through the chambers with blower fans (for details of chamber setup see (20)). Four control chambers were maintained at ambient atmospheric CO\(_2\) (360 ppm; AMB treatment), while four were maintained at an elevated level of CO\(_2\) (720 ppm; ELEV treatment) by dispensing 100% CO\(_2\) into the blower fans. Elevated CO\(_2\) concentrations were maintained by continuously monitoring CO\(_2\) in each of the ELEV chambers and one of the AMB chambers with an LI-6252 infrared gas analyzer (LI-COR, Lincoln, NE) and periodically adjusting the flow of CO\(_2\) into the chambers as needed. Treatments were maintained throughout the growing season each...
year from May until leaf senescence in November. During the treatment periods, all chambers were watered twice weekly with equal volumes provided to each tree. Well water from UMBS, which contained non-detectable levels of total inorganic nitrogen and orthophosphorus (R. VandeKopple, UMBS Resident Biologist, unpublished data) was used for watering. In Fall 2003 leaves were collected after abscission and air dried.

**Chemical Analysis of Leaves**

Simple phenolic content (monomeric phenolic compounds) was determined by the Folin Ciocalteu method (51). Lignin content was determined by the rapid microscale acetyl bromide-based method (15). Carbon and nitrogen concentrations were determined using a Costech Elemental Analyzer Model 4010 (Analytical Technologies, Inc., Valencia, CA). Condensed tannin content was determined by the method of Hagerman (37). The reference material for the condensed tannin assay was extracted from Aspen leaves gathered from the field site. Because reactivity of condensed tannins varies by species, the results should only be used as an index of relative treatment responses and not an expression of absolute amounts.

Leaf chemistry data were analyzed by two-way analysis of variance (ANOVA) using Systat version 12 (Systat Software, Inc., San Jose, CA). For significant effects, pairwise comparisons were based on Fisher’s LSD test.

**Stream Incubation**

One gram (air dry weight) of whole leaves was placed into individual 1.4-mm-mesh bags, with one leaf-species-treatment combination per bag and five replicate bags
for each leaf-species-treatment combination. Bags were strung on monofilament line in
random order and suspended in the East Branch of the Maple River on November 1,
2003. Bags were suspended below the water surface and above the stream bottom by
attaching the monofilament line to metal posts driven into the stream bed. The East
Branch of the Maple River (45°34.505′N, 84°44.706′W), located near Pellston, Michigan,
is a third order headwater stream that flows through undeveloped wetlands and northern
hardwood forests. Nitrogen and phosphorus concentrations are generally low in the
stream, with a mean nitrate concentration of 10.6 µg N·l⁻¹, a mean ammonia concentration
of 23.4 µg N·l⁻¹, and a mean soluble reactive phosphorus concentration of 2.2 µg P·l⁻¹ at
the study site (J.A. Teeri unpublished data). Mean stream width in the study reach was 5
m and mean depth was 0.5 m. Leaf bags were collected after 14 days in the stream and
frozen for transport to Loyola University Chicago.

Microbial Community Size

Living biomass of the fungal communities colonizing the leaves was estimated
from concentrations of ergosterol (35). Leaves were subsampled using a sterile cork borer
with a 2 cm diameter. Leaf disks were placed in HPLC grade methanol and stored in a
freezer until analyzed. Ergosterol in samples was extracted, partially cleaned by solid-
phase extraction (35), and quantified by a Shimadzu High Pressure Liquid
Chromatography (HPLC) system. Ergosterol concentrations were converted to fungal
biomass assuming a conversion factor of 5 µg ergosterol/mg fungal dry mass (33).
Additional leaf disks were collected and dried at 105°C overnight in order to determine
fungal biomass concentrations per gram dry weight of leaf material.
The number of bacterial cells colonizing leaves was determined by direct epifluorescence microscopy after staining with SYBR Gold (Invitrogen, Carlsbad, CA). Subsamples of leaf litter (2 cm diameter leaf discs) were preserved in a filtered (0.2 µm) solution of 3.7 % formaldehyde and 0.1 M tetrasodium pyrophosphate and stored at 4°C until analyzed. Bacterial cells attached to leaf samples were detached by ultrasonic probe sonication for 30 sec on ice. After sonication, sample aliquots were stained and filtered onto 0.2 µm Anodisc filter membranes (Whatman, Piscataway, NJ). Filters were mounted on glass slides and bacterial cells were enumerated in a minimum of 10 fields using a BH2 epifluorescence microscope (Olympus, Center Valley, PA). Additional leaf disks were collected and dried at 105°C overnight in order to determine bacterial cell densities per dry weight of leaf material.

Fungal biomass and bacterial counts data were analyzed by two-way analysis of variance (ANOVA) using Systat version 12 (Systat Software, Inc., San Jose, CA). For significant effects, pairwise comparisons were based on Fisher’s LSD test.

**Molecular Analysis of Microbial Communities**

*DNA Isolation*

Genomic DNA was isolated from each leaf pack using the Ultra Clean Soil DNA Kit (MoBio Laboratories, Salana Beach, CA). This genomic DNA was used for terminal restriction fragment length polymorphism (T-RFLP) analysis and clone library analysis, as described below.
Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis

Fungal intergenic transcribed spacer (ITS) regions were amplified by PCR using primers ITS1F and ITS4 (30). ITS4 was synthesized by Operon (Alameda, CA) and ITS1F was synthesized and labeled at the 5’ end with dye IRD-800 by LI-COR (Lincoln, NE). Each 25 µL PCR reaction contained 0.4 µM forward primer, 4.0 µM reverse primer, 200 µM deoxynucleoside triphosphates (Amersham Biosciences, Piscataway, NJ), 1X PCR buffer (Promega, Madison, WI), 2.5 mM MgCl₂ (Promega), 1.5 units of Taq DNA polymerase (Promega), and 1.0 µL of template DNA. For these samples a 1:10 dilution of DNA extract was found to be optimal for amplification. PCR reactions were run in a PTC-100 DNA thermal cycler (MJ Research, Waltham, MA). Cycling parameters were as described previously (30). Two replicate PCR reactions were run for each sample and the products were pooled in order to produce enough product for T-RFLP analysis.

Bacterial 16S rRNA genes were amplified by polymerase chain reaction (PCR) using primers 8F and 926R (48). Primer 926R was synthesized by Operon, and 8F was synthesized and labeled at the 5’ end with dye IRD-800 by LI-COR. PCR reactions were set up as described above for fungal community analysis. Cycling parameters were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15s, annealing at 56.4°C for 20s, and extension at 72°C for 30s, followed by a final extension step for 3 min at 72°C. Two replicate PCR reactions were run for each sample and the products were pooled in order to produce enough product for T-RFLP analysis.
PCR products were purified using the Ultra Clean PCR Clean-up DNA Purification Kit (MoBio Laboratories) and quantified based on analysis of agarose gel band intensities using Quantity One Software (BioRad, Hercules, CA). PCR products (25 ng) were digested individually with Msp I and Alu I (for fungi) and Hae III, Msp I, and Mse I (for bacteria). All restriction enzymes were obtained from New England BioLabs (Beverly, MA) and were utilized according to the manufacturer’s instructions. To verify complete digestion, DNA isolated from pure culture controls was amplified and digested in parallel with leaf samples.

After denaturation at 94°C for 2 min., digested DNA samples were electrophoresed on a 5.5% acrylamide gel using DNA Sequencer Model 4000L (LI-COR). The sizes of the TRFs were determined using Quantity One Software (BioRad). Each TRF was scored as present or absent, and any TRFs present in less than two samples were excluded from data analysis. The resultant fungal and bacterial T-RFLP data sets were analyzed by non-metric multi-dimensional scaling (MDS) using the Primer V.5 software package (Primer-E Ltd, Plymouth, UK). For a full description of the MDS procedure see Clarke and Warwick (19). Briefly, each T-RFLP data set was imported into Primer V.5 and a similarity matrix was calculated using the Bray-Curtis coefficient (11). The MDS procedure was then used to ordinate the similarity data following 100 random restarts. The analysis of similarity (ANOSIM) routine in Primer V.5 was used to determine if there were statistically significant differences between groups of samples. ANOSIM reports R-statistics and statistical significance of the R-statistics (p-values). An R-statistic close to 1 indicates that there is greater similarity for samples within groups than there is for samples between groups. An R-statistic close to 0 indicates that that the
similarity for samples within groups is not greater that the similarity for samples between
groups. The $p$ value reflects the statistical significance of the R statistic (19).

Clone Library Analyses

Fungal and bacterial clone libraries were prepared from one Aspen AMB leaf
pack and one Aspen ELEV leaf pack. Fungal ITS sequences were amplified by PCR (as
described above) using unlabeled primers ITS1F and ITS4 (30). Bacterial 16S rRNA
genes were amplified by PCR (as described above) using unlabeled bacterial domain
primers 11F (42) and 926R (48). All primers for clone library construction were obtained
from Operon. PCR amplicons were cloned with the TOPO-TA cloning kit (Invitrogen,
Carlsbad, CA) using vector pCR4 and transformed into chemically competent
*E. coli.*

Transformed *E. coli* were grown overnight on LB agar plates containing 50 µg/ml
kanamycin. Randomly selected colonies were transferred to LB broth containing 50
µg/ml kanamycin and grown overnight at 37°C. Plasmids were isolated using either the
Mini Plasmid Prep Kit (MoBio Laboratories) or the QIAprep Spin Miniprep Kit (Qiagen,
Valencia, CA) and PCR-screened for the presence of inserts of appropriate size using
M13F and M13R primers. Each plasmid that contained an appropriately sized insert was
sequenced bi-directionally using M13F and M13R primers by the University of Chicago
Cancer Research Center’s DNA Sequencing Facility (Chicago, IL).

The SeqMan component of the Lasergene software package (DNASTAR, Inc.,
Madison, WI) was used to assemble a consensus sequence for each clone. Fungal
sequences were deposited to Genbank under accession numbers GU065446 to
GU065632. Bacterial sequences were deposited to Genbank under accession numbers
GU568764 to GU569021. Clone sequences were identified by comparison to the Genbank nucleotide database using BLAST (3) via the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/). BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) was used to align each clone library via Clustal W and calculate a distance matrix. OTUs were established using MOTHUR (60) based on the furthest neighbor model and were defined by 3% distance. Collector’s curves of the Chao1 richness estimator (16) and the Shannon Diversity Index were plotted using MOTHUR. Venn diagrams were generated using MOTHUR and diagrammed using the DrawVenn Application (17).

Results

Leaf Chemistry

Two-way ANOVA revealed that ELEV treatment of trees had some significant impacts on leaf chemistry (Table 1). Specifically, there were significant increases in simple phenolic content (p<0.001), condensed tannin content (p<0.001) and C:N ratio (p<0.001) of leaves with ELEV treatment. In contrast, there were no significant effects of ELEV treatment on lignin content of leaves. Leaf chemistry also varied significantly between tree species (Table 1). There was a significant effect of tree species on simple phenolic content (p<0.001) of leaves, with Maple leaves having significantly higher simple phenolic content than Aspen (p<0.001) and Willow (p<0.01) leaves. There was also a significant effect of tree species on lignin content of leaves (p<0.05) with Aspen leaves having significantly higher lignin content than Maple (p<0.05) and Willow leaves (p<0.05). Finally, there was a significant effect of tree species (p<0.001) on C:N of

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leaves, with Maple leaves having significantly higher C:N than Aspen or Willow leaves (p<0.001), and no significant difference in C:N between Aspen and Willow leaves. Two way ANOVA revealed no significant interactions between treatment and tree species for simple phenolic content, lignin content, and C:N ratio.

Microbial Community Size

Two way ANOVA revealed a significant effect of ELEV treatment on fungal biomass (p<0.05), with ELEV leaves on average showing lower fungal biomass than AMB leaves (Fig. 1A). Fisher’s LSD test showed that the decrease in fungal biomass with ELEV treatment was significant for Aspen leaves (p<0.01) but was not significant for Maple or Willow leaves. Two-way ANOVA also revealed a significant effect of tree species on fungal biomass (p<0.01) with Maple leaves supporting significantly less fungal biomass than Aspen (p<0.01) or Willow (p<0.005) leaves, but no significant difference in fungal biomass between Aspen and Willow leaves. Two way ANOVA revealed no significant interaction between treatment and tree species for fungal biomass.

Two-way ANOVA revealed no significant effect of ELEV treatment on bacterial counts (Fig. 1B). However, one-way ANOVA for individual tree species demonstrated that ELEV treatment resulted in a significant decrease in bacterial counts on Aspen leaves (p<0.05) but no significant change in counts for Maple or Willow leaves. Also, two-way ANOVA revealed no significant effect of tree species and no significant interaction between treatment and tree species for bacterial counts.

Microbial Community Composition
MDS analysis of fungal T-RFLP data revealed no clear separation of AMB vs. ELEV samples across all tree species (Fig. 2A) and ANOSIM analysis showed a low R value (0.018) for the comparison of all AMB vs. all ELEV samples (Table 2). However, when tree species were examined individually, there was a clear separation of AMB Aspen samples from ELEV Aspen samples (Fig. 2A) as well as a high R value (0.584) that was significant (p<0.01) for the comparison of AMB Aspen vs. ELEV Aspen samples (Table 2). In contrast, MDS and ANOSIM analyses did not reveal significant differences in fungal communities on Maple or Willow leaves based on ELEV treatment. Finally, MDS analysis of fungal T-RFLP data revealed a clear separation of samples based on tree species (Fig. 2A) and ANOSIM analysis showed high and significant R values for the comparisons of samples by tree species (Table 2).

MDS analysis of bacterial T-RFLP data also revealed no clear separation of AMB vs. ELEV samples across all tree species (Fig. 2B) and ANOSIM analysis showed a low R value (0.001) for the comparison of all AMB vs. all ELEV samples (Table 2). However, when tree species were examined individually, there was a clear separation of AMB Aspen samples from ELEV Aspen samples (Fig. 2B) as well as a high R value (0.778) that was significant (p<0.10) for the comparison of AMB Aspen vs. ELEV Aspen samples (Table 2). In contrast, MDS and ANOSIM analyses did not reveal significant differences in bacterial communities on Maple or Willow leaves based on ELEV treatment. Finally, MDS analysis of bacterial T-RFLP data revealed no clear separation of samples based on tree species when AMB and ELEV samples were taken together (Fig. 2B) and ANOSIM analysis showed low R values for the comparisons of samples by species (Table 2). However, the MDS graph did show a separation of samples by tree.
species when only the AMB samples were compared (Fig. 2B) and there were significant high R values for the comparisons of Aspen AMB vs. Maple AMB and Aspen AMB vs. Willow AMB, respectively. Thus, there were some significant differences in bacterial community composition based on tree species, but these differences were obscured by ELEV treatment.

Since the T-RFLP data revealed significant differences in fungal and bacterial communities with ELEV treatment only for Aspens leaves (see Fig. 2 and Table 2), fungal ITS and bacterial 16S rRNA gene clone libraries were assembled and sequenced for Aspen AMB and ELEV leaves. A total of 187 fungal clones (92 from AMB and 95 from ELEV) and a total of 258 bacterial clones (132 from AMB and 126 from ELEV) were collected and sequenced. The fungal clone libraries produced a total of 15 OTUs at 3% distance (Fig. 3A). Collectors curves for the total estimated number of fungal OTUs based on the Chao1 richness estimator showed that OTU estimates were unchanged for the last 10-15 clones in each library (Fig 4A), indicating that the fungal clone libraries were of sufficient size to provide an accurate estimate of the total OTU richness (59). Although there were slightly more fungal OTUs found on the ELEV leaves (13 OTUs) than on the AMB leaves (9 OTUs) (Fig. 3A), comparison of the Chao1 values indicated that the ELEV community was estimated to contain 26 OTUs with a 95% confidence interval of 15 to 79 and the AMB community was estimated to contain 11 OTUs with a 95% confidence interval of 8 to 33 (Fig. 4A), thus indicating that there was not a significant difference in the estimated number of fungal OTUs in the AMB and ELEV communities. In addition, collectors curves based on the Shannon Diversity index revealed no significant difference in OTU diversity between AMB and ELEV fungal
communities (Fig. 5A). These Shannon index collectors curves were also fairly stable over the last 20-30 clones in each library, suggesting that the fungal clone libraries were of sufficient size to provide an accurate estimation of the diversity of these communities.

There were 7 fungal OTUs that were common to both AMB and ELEV leaves, 2 fungal OTUs that were unique to AMB leaves, and 6 fungal OTUs that were unique to ELEV leaves, suggesting a significant shift in community composition (Fig. 3A).

However, the vast majority of fungal ITS sequences (93%) were in OTUs that were common to AMB and ELEV leaves. Among the sequences that were found in both AMB and ELEV leaves, 72% were in an OTU that showed 98 to 100% identity to the genus *Cladosporium*, 17% were in an OTU that could not be identified by BLAST analysis (no matches with more than 86% identity), and 5% were in an OTU that showed 100% identity to *Alternaria alternata*. Only 4% of the fungal ITS sequences were unique to the AMB leaves, and these were found in two OTUs, one showing 99% identity to *Aureobasidium pullulans* and one showing 90% identity to organisms from the *Cladosporium* genus. Finally, only 3% of the fungal ITS sequences were unique to the ELEV leaves, and these were found in six different OTUs. Two of the ELEV-specific OTUs were identified via BLAST analysis as *Mycosphaerella punctiformis* (94% identity) and *Leptosphaeriaceae* spp. (94% identity), while the remaining ELEV-specific OTUs did not show significant matches (i.e. > 90% identity) via BLAST.

The bacterial clone libraries produced a total of 107 OTUs at 3% distance (Fig. 3B). Collectors curves for the total estimated number of bacterial OTUs based on the Chao1 richness estimator were fairly stable over the last 10 clones in each library (Fig 4B), indicating that the bacterial clone libraries were of sufficient size to provide a
reasonable estimation of the total OTU richness (59). There were more bacterial OTUs found on the ELEV leaves (77 OTUs) than on the AMB leaves (59 OTUs) (Fig. 3B). Comparison of the Chao1 number of bacterial OTUs also showed a higher number of estimated OTUs on the ELEV leaves (200 OTUs) as compared to the AMB leaves (107 OTUs), but the confidence intervals indicated that these differences were not statistically significant (Fig. 4B). However, collectors curves based on the Shannon Diversity index showed significantly higher bacterial diversity for the ELEV leaves as compared to the AMB leaves (Fig. 5B). In addition, the Shannon index collectors curves were fairly flat once each of the libraries exceeded 100 clones, suggesting that the bacterial clone libraries were large enough to provide an accurate estimation of the diversity of these communities.

The distribution of bacterial OTUs between the AMB and ELEV leaves revealed a dramatic shift in the composition of the bacterial communities based on ELEV treatment, with both the AMB and ELEV bacterial communities containing more unique OTUs than shared OTUs (Fig. 3B). These data supported the shift in bacterial community composition for Aspen leaves that was revealed by T-RFLP analysis (Fig. 2B). Analysis of the OTUs present in each of the clone libraries showed that ELEV treatment resulted in a relative increase in the proportion of the clone libraries represented by alphaproteobacteria (from 23% to 29%) and a relative decrease in betaproteobacteria (from 30% to 22%) as compared to the AMB treatment. In addition, the ELEV clone library showed a higher proportion of cytophaga-flavobacter-bacteroides (CFB) group bacteria (35% as compared 20%) and a much lower proportion of firmicutes (2% as compared to 21%) than the AMB clone library. This drop in firmicutes with ELEV
treatment was noteworthy because this is the only one of the dominant phyla that is Gram positive. In total, 23% of the sequences in the AMB clone library corresponded to Gram positive bacteria, whereas only 4% of the sequences in the ELEV clone library corresponded to Gram positive bacteria.

Discussion

Growth of trees under elevated atmospheric carbon dioxide had some significant impacts on leaf chemistry, including increases in simple phenolics, condensed tannins, and C:N ratio. These results agree with previous studies that have demonstrated changes in leaf chemistry in response to elevated carbon dioxide in several tree species including Populus tremuloides and Acer saccharum (both used in this study) and Quercus rubra (47, 70). However, none of the leaf species in our study showed significant changes in lignin content with ELEV treatment. This was somewhat surprising as Rier et al. (58) had observed a significant increase in lignin content in Aspen leaves grown under elevated atmospheric carbon dioxide using the same experimental design that was used in our study. The lignin content of Aspen leaves did increase with ELEV treatment in our study, but this increase was not statistically significant (p=0.374). In contrast, both Maple and Willow leaves showed decreases in lignin content with ELEV treatment, but again these decreases were not statistically significant (p=0.197 and 0.371, respectively).

The chemical changes observed in leaves with ELEV treatment could have significant implications for forested headwater streams, as these ecosystems are dependent on leaf litter as a major source of carbon (29). Aquatic invertebrates feed on leaf detritus in these streams, and increases in phenolics and tannins have been shown to
reduce herbivory/detritivory on plant tissues (27). Likewise, increased C:N ratio of leaves makes them less nutritious for invertebrate consumers (12). The chemical changes in leaves resulting from ELEV treatment also have the potential to impact microbial communities. Previous studies comparing the decomposition rates of various leaf species have demonstrated that leaves that have naturally high concentrations of phenolic compounds and lignin and are relatively low in nitrogen content (e.g. Oak) support less bacterial biomass and are more slowly decomposed in streams whereas species that exhibit lower concentrations of these compounds and relatively higher nitrogen contents (e.g. Alder, Maple) tend to support more bacterial biomass and are more rapidly decomposed (22, 56). Similarly, polyphenolic compounds have been shown to slow the growth of both bacteria and fungi on leaf litter (10). Therefore, we would expect lower quality leaves to support less microbial growth, a prediction that was supported by our results. For example, Maple leaves had significantly higher simple phenolic content and a significantly higher C:N than Aspen and Willow leaves, and Maple leaves supported significantly lower fungal biomass than Aspen or Willow leaves. In addition, ELEV treatment lowered the quality of Aspen leaf litter and we observed significantly lower fungal biomass and bacterial counts on ELEV Aspen leaves. This result is in agreement with previous data for Aspen leaves treated with elevated atmospheric carbon dioxide (58). Previous studies have shown that bacterial and fungal growth on decomposing leaves in aquatic ecosystems is synergistic, with each group growing faster in the presence of the other group (10). However, antagonistic effects have also been observed (54). Whether the bacterial-fungal interactions in our study were synergistic or antagonistic, the decreases in fungal biomass and bacterial counts that were observed

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were likely interrelated, with a decrease in one microbial group possibly influencing
growth of the other group.

The decrease in microbial colonization of Aspen leaves grown under ELEV
conditions could have significant food web implications. Invertebrate consumers in
headwater streams are dependent on microbes colonizing leaf detritus both as a source of
nutrition (23) and for their role in breaking down indigestible leaf components (6). Lower
microbial colonization of leaves should make leaf detritus less nutritious for
invertebrates, and indeed previous work has demonstrated that microbially colonized
Aspen leaves produced under elevated atmospheric carbon dioxide resulted in decreased
growth rates in cranefly larvae (*Tipula abdominalis*) (70), increased mortality rates in one
species of mosquito larvae (*Aedes albopictus*), and decreased development rates for
several species of mosquito larvae (*A. triseriatus, A. aegypti and Armigeres subalbatus*)
(69). The fact that we observed decreased microbial colonization of ELEV Aspen leaves
is especially significant for our study site since Aspen (*Populus tremuloides*) is the most
abundant tree species in Michigan (61) and it accounts for approximately 22% of the leaf
litter in our study stream (Tuchman unpublished data).

Interestingly, the ELEV treatment in our study did not have a significant effect on
microbial colonization of Maple or Willow leaves despite the fact that the quality of
Maple and Willow leaves was negatively impacted by ELEV treatment. The contrasting
microbial responses for Aspen, Maple and Willow leaves could be related to the fact that
Aspen leaves had significantly higher lignin content that Maple or Willow leaves,
suggesting that the high lignin content in combination with the other observed chemical
changes had a negative effect on bacterial and fungal communities. Alternatively, ELEV
treatment may have resulted in species-specific changes in some aspect of leaf chemistry that was not measured in this study. Further work will be necessary to clarify this issue.

No previous study we are aware of has examined the effects of tree growth under elevated atmospheric carbon dioxide on the composition of microbial communities colonizing leaf litter in streams. In our study T-RFLP analysis of fungal ITS sequences revealed a statistically significant change in fungal community composition in response to ELEV treatment for Aspen leaves, but not for Maple or Willow leaves. This tree-species-specific result followed the pattern observed for fungal biomass (i.e. a change with ELEV treatment for Aspen leaves but not for Willow or Maple leaves). However, analysis of fungal ITS clone libraries suggested that the shift in fungal community composition on Aspen leaves that was indicated by T-RFLP was actually driven by a relatively small fraction of the fungal communities. Specifically, of the 92 fungal ITS sequences collected from the AMB Aspen leaves only 7 sequences (i.e. 8%) were unique to the AMB leaves, and for the ELEV leaves only 6 out of 95 ITS sequences collected (i.e. 6%) were unique to the ELEV leaves. The apparent conflict between the fungal T-RFLP and clone library results was due to the fact that the T-RFLP analysis was based on presence-absence of individual TRFs, so it did not account for differences in the numbers of individuals within each ribotype. In contrast, the clone library analysis did provide insight into the number of individual sequences within each OTU, and analysis of the clone library data suggested that the fungal communities colonizing Aspen leaves were predominantly stable despite ELEV treatment and the resultant changes in leaf chemistry. This result was somewhat surprising given the fact that ELEV treatment resulted in lower fungal biomass on Aspen leaves. We would have expected the changes in leaf chemistry
that inhibited fungal growth to have exerted some selective pressure and resulted in a shift in fungal species composition, but based on clone library analysis this does not appear to have occurred to a significant extent.

The most numerically dominant fungal OTU on both AMB and ELEV Aspen leaves, which accounted for 67% of the fungal ITS sequences collected, was identified as corresponding to *Cladosporium sp*. The dominance of Cladosporia was not surprising as this is one of the most common fungal groups isolated from dead organic material in terrestrial and aquatic environments (25) and it has been identified previously on leaf litter decomposing in streams (7, 14, 14). However, numerous studies have identified aquatic hyphomycetes as the dominant fungal group on decomposing leaf litter in streams (for review see (34)). The general consensus is that Cladosporia, which are common phylloplane fungi, are most likely present on leaves before they enter a stream. Once the leaves enter a stream the Cladosporia can persist, but are usually replaced by aquatic hyphomycetes over a period of a few weeks (24). Therefore, it was somewhat surprising that we did not find any ITS sequences corresponding to aquatic hyphomycetes on either the AMB or ELEV Aspen leaves. It is possible that our in-stream incubation was not long enough to allow aquatic hyphomycete colonization. However, the 14 day incubation time was chosen based on previous work by our group at this site, which demonstrated that 14 days was adequate to allow the accumulation of significant bacterial and fungal biomass on Aspen leaves (58). Data in the literature also suggested that this incubation time would be appropriate, as previous studies have demonstrated that aquatic hyphomycetes colonize leaves rapidly after deposition, germinate within 2-6 hours, and start to release conidia in as little as 6-10 days (34). In addition, 14 days was chosen for this study in
order to capture the period of highest microbial activity, as previous work by our group at this site indicated that both bacterial productivity and microbial respiration on Aspen leaves peaked between 10 and 15 days and then began to decline (58) and others have found that fungal growth rate and production on decomposing leaves peak rapidly following leaf deposition (34).

Another possible explanation for the absence of aquatic hyphomycete sequences in our clone libraries is that their growth may have been limited by the oligotrophic conditions in the Maple River. Numerous studies have demonstrated the significant impact of stream nutrient concentrations on fungal biomass, growth rates, and rates of litter decomposition (for review see (34)). Despite the lack of aquatic hyphomycete sequences, the data collected in our study are still relevant to ecosystem function, as significant invertebrate colonization of leaf detritus in Midwestern streams can occur within the first 14 days after deposition (68). In addition, several recent studies have suggested that Cladosporia can make significant contributions to leaf decomposition in streams. For example, Baschien et al. (8) demonstrated that *Cladosporium herbarum* was the dominant fungal species on willow leaves collected from a stream in northern Germany, and they demonstrated that *C. herbarum* showed cellulolytic activity when incubated with leaf litter in aquatic microcosms.

Our fungal T-RFLP data also revealed a clear separation of fungal communities based on tree species. This result contradicts the results of Nikolcheva and Bärlocher (55) who examined stream fungal communities colonizing leaves from three tree species that differed in chemical composition and showed no effect of leaf species type on fungal community composition based on DGGE and T-RFLP analyses of fungal ITS sequences.
However, Nikolcheva and Bärlocher used different tree species than those used in our study, so the differing results may reflect differences in the tree species used. Another possible explanation for these different results may relate to the nutrient levels in the streams. The stream in Nova Scotia that was used by Nikolcheva and Bärlocher contained a 100-fold higher concentration of nitrate than our study stream, the Maple River. The low levels of nitrate and ammonium in the Maple River may have exacerbated the effects of differences in C:N between the different leaf species used in our study, as it would have been more difficult for fungi to import nitrogen from the stream water to compensate for leaves with high C:N.

In our study T-RFLP analysis demonstrated that ELEV treatment resulted in a dramatic shift in bacterial community composition for Aspen leaves, but not for Maple or Willow leaves. This shift in community composition on Aspen leaves was supported by clone library analysis of bacterial 16S rRNA genes. This tree-species-specific result followed the pattern observed for bacterial counts (i.e. a change with ELEV treatment for Aspen leaves but not for Willow or Maple leaves). Clone library analysis also suggested that ELEV treatment resulted in an increase in bacterial species diversity on Aspen leaves. This shift in species composition and increase in diversity was likely the result of the significant chemical changes that occurred in Aspen leaves with ELEV treatment. These chemical changes may have limited the growth of the normally dominant bacterial groups and opened up opportunities for other bacterial groups. In addition, the reduced fungal biomass that was observed on ELEV Aspen leaves may have had an impact on the bacterial species present, as bacterial and fungal growth on decomposing leaves in aquatic ecosystems has been shown to be potentially synergistic (10) or antagonistic (54).
The bacterial groups identified on Aspen leaves in our study are in general agreement with previous studies, which have found proteobacteria and CFB to be the dominant bacterial groups on leaves decomposing in streams (22, 53, 67). The increase in CFB that we observed with ELEV treatment may have been related to the chemical changes that occurred in Aspen leaves grown under elevated CO$_2$, as many species within the CFB are known to degrade complex biopolymers and to be important contributors to the breakdown of lignocellulosic plant materials (50, 52). The dramatic decrease we observed in Gram positive bacteria with ELEV treatment may also have been related to changes in leaf chemistry, specifically the increase in C:N ratio, as several studies have demonstrated that Gram positive organisms respond negatively to high C:N ratios on decomposing plant material (28) and in soils (38, 40, 74). It is important to note that the impacts of leaf C:N on bacterial communities observed in our study may have been magnified by the low nitrogen concentration in our study stream, which would have made it more difficult for bacteria to import nitrogen from the stream water to compensate for the high C:N ratio of the ELEV Aspen leaves.

The results of our study have demonstrated that growth of trees under elevated atmospheric carbon dioxide can result in shifts in the composition of microbial communities colonizing leaf litter in temperate woodland streams, and that these community shifts are likely related to changes in leaf composition. Further work is needed to elucidate the possible food web implications of these shifts in microbial community composition.
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Figure Legends

Figure 1. Fungal biomass (A) and direct counts of bacterial cells (B) on ambient CO2 leaves (white bars) and elevated CO2 leaves (gray bars). Each bar represents mean value (n=4) and error bars represent standard error. Significant differences between ambient and elevated treatments (p<0.05) indicated by (*).

Figure 2. Non-metric multidimensional scaling analysis of fungal (A) and bacterial (B) T-RFLP data. Aspen ambient CO2 leaves (open squares), Aspen elevated CO2 leaves (gray squares), Maple ambient CO2 leaves (open circles), Maple elevated CO2 leaves (gray circles), Willow ambient CO2 leaves (open triangles), Willow elevated CO2 leaves (gray triangles).

Figure 3. Venn diagrams showing shared and unique fungal (A) and bacterial (B) OTUs (3%) based on clone libraries from ambient CO2 Aspen leaves (AMB) and elevated CO2 Aspen leaves (ELEV).

Figure 4. Collectors curves for Chao1 estimated total numbers of fungal (A) and bacterial (B) OTUs (3% distance) based on clone libraries from ambient CO2 Aspen leaves (gray line) and elevated CO2 Aspen leaves (black line). Error bars represent 95% confidence interval.
Figure 5. Collectors curves for Shannon Diversity Index scores for fungal (A) and bacterial (B) clone libraries from ambient CO2 Aspen leaves (gray line) and elevated CO2 Aspen leaves (black line). Error bars represent 95% confidence interval.
Table 1. Chemical analyses of leaves

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>Simple Phenolics (%)†</th>
<th>Condensed Tannins (%)†</th>
<th>Lignin (%)†</th>
<th>C:N Ratio ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspen</td>
<td>Ambient</td>
<td>6.8 (0.0)</td>
<td>10.3 (0.4)</td>
<td>17.8 (3.4)</td>
<td>70.8 (4.9)</td>
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<tr>
<td>Aspen</td>
<td>Elevated</td>
<td>7.9 (0.3) *</td>
<td>16.5 (2.0) *</td>
<td>21.6 (1.8)</td>
<td>156.1 (26.7) *</td>
</tr>
<tr>
<td>Maple</td>
<td>Ambient</td>
<td>8.4 (0.9)</td>
<td>11.0 (0.6)</td>
<td>16.1 (1.0)</td>
<td>194.9 (11.0)</td>
</tr>
<tr>
<td>Maple</td>
<td>Elevated</td>
<td>10.9 (0.4) *</td>
<td>20.4 (2.6) *</td>
<td>13.7 (1.2)</td>
<td>236.8 (25.5) *</td>
</tr>
<tr>
<td>Willow</td>
<td>Ambient</td>
<td>6.0 (0.3)</td>
<td>19.5 (1.3)</td>
<td>15.7 (1.1)</td>
<td>92.5 (0.9)</td>
</tr>
<tr>
<td>Willow</td>
<td>Elevated</td>
<td>7.3 (0.3) *</td>
<td>24.5 (2.3) *</td>
<td>14.1 (1.1)</td>
<td>147.3 (7.9) *</td>
</tr>
</tbody>
</table>

Each data point represents mean (n=3) with standard error values in parentheses.

† Percentage values reflect percentage of leaf dry mass.
‡ Molar ratio
* significant difference with ELEV treatment (p<0.001).
Table 2. ANOSIM analyses for bacterial and fungal T-RFLP data

<table>
<thead>
<tr>
<th>Selected Comparisons</th>
<th>Bacteria</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R Statistic</td>
<td>p value</td>
</tr>
<tr>
<td>Global†</td>
<td>0.381</td>
<td>0.001</td>
</tr>
<tr>
<td>All Ambient vs All Elevated</td>
<td>0.001</td>
<td>0.040</td>
</tr>
<tr>
<td>Aspen Ambient vs Aspen Elevated</td>
<td>0.778</td>
<td>0.100</td>
</tr>
<tr>
<td>Maple Ambient vs Maple Elevated</td>
<td>0.130</td>
<td>0.200</td>
</tr>
<tr>
<td>Willow Ambient vs Willow Elevated</td>
<td>-0.333</td>
<td>1.000</td>
</tr>
<tr>
<td>All Aspen vs All Maple</td>
<td>0.207</td>
<td>0.050</td>
</tr>
<tr>
<td>All Aspen vs All Willow</td>
<td>0.307</td>
<td>0.020</td>
</tr>
<tr>
<td>All Maple vs All Willow</td>
<td>0.348</td>
<td>0.009</td>
</tr>
<tr>
<td>Aspen Ambient vs Maple Ambient</td>
<td>0.741</td>
<td>0.100</td>
</tr>
<tr>
<td>Aspen Ambient vs Willow Ambient</td>
<td>1.000</td>
<td>0.100</td>
</tr>
<tr>
<td>Maple Ambient vs Willow Ambient</td>
<td>0.111</td>
<td>0.400</td>
</tr>
</tbody>
</table>

† Global test is based on the null hypothesis that there are no differences between any of the groups.
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