

Alteration of Microbial Communities Colonizing Leaf Litter in a Temperate Woodland Stream by Growth of Trees under Conditions of Elevated Atmospheric CO₂[∇]

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Received 27 January 2010/Accepted 28 May 2010

Elevated atmospheric CO₂ 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 they are detritus-based ecosystems that rely on leaf litter as their primary source of organic carbon. Fungi and bacteria play key roles in the entry of terrestrial carbon into 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 CO₂ 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) CO₂, and their leaves were incubated in a woodland stream. Elevated-CO₂ treatment resulted in significant increases in the phenolic and tannin contents 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 terminal restriction fragment length polymorphism (T-RFLP) and clone library sequencing revealed that fungal community composition was mostly unchanged by the elevated-CO₂ 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 cytophaga-flavobacter-bacteroides (CFB) group sequences and decreased numbers of betaproteobacterial and firmicutes sequences, as well as a pronounced decrease in overall Gram-positive bacterial sequences.

The concentration of atmospheric CO₂ 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 CO₂ is believed to be due to the extensive use of fossil fuels and changes in land use patterns (5). Elevated atmospheric CO₂ 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 CO₂ 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 CO₂ is unlikely to have direct impacts on forested headwater streams, as they are primarily heterotrophic systems (2) in which CO₂ 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 the microbial biomass (64, 66). In addition, microbes convert indigestible leaf components (e.g., lignin and cellulose) into 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

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[∇] Published ahead of print on 11 June 2010.

illustrated by a number of studies comparing decomposition of leaves from different tree species (for a review, see reference 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). The leaf carbon-to-nitrogen (C/N) ratio also impacts decomposition rates; leaf litter with a high C/N ratio tends to decompose more slowly than litter with a low C/N ratio (62). These trends are relevant to atmospheric CO₂ concentrations because elevated atmospheric CO₂ has been shown to increase the concentrations of phenolic compounds (lignin and tannins), as well as the C/N ratio of leaves of C3 plants (31, 46, 58, 70). Therefore, it is reasonable to hypothesize that growth of trees under elevated CO₂ 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 CO₂ 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 CO₂ could impact microbial community composition. Several studies have demonstrated that the compositions 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 CO₂ on the compositions 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 abilities to degrade various components of leaf litter (1, 67), so the species compositions 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 (9, 32), and thus, they may differ in their nutritional values to aquatic invertebrates.

In the current study, we tested the hypothesis that changes in leaf chemistry caused by elevated CO₂ would result in changes in the biomass and composition of detrital microbial communities by growing three tree species under ambient or elevated CO₂, 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 CO₂. *P. tremuloides* (common name, quaking aspen, here referred to as aspen), *Salix alba* (common name, white willow, here referred to as willow), and *Acer saccharum* (common name, sugar maple, here 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, 2-year-old willow clones, and 2-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 those of 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 by 6-foot-high cylindrical clear plastic open-top chambers. Air was circulated through the chambers with blower fans (for details of the chamber setup, see reference 20). Four control chambers were maintained at ambient atmospheric CO₂ (360 ppm; AMB treatment), while four were maintained at an elevated level of CO₂ (720 ppm; ELEV treatment) by dispensing 100% CO₂ into the blower fans. Elevated CO₂ concentrations were maintained by continuously monitoring the CO₂ 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 CO₂ into the chambers as needed. The 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 nondetectable levels of total inorganic nitrogen and orthophosphorus (R. VandeKopple, UMBS resident biologist, unpublished data) was used for watering. In the fall of 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). The condensed-tannin content was determined by the method of Hagerman and Butler (37). The reference material for the condensed-tannin assay was extracted from aspen leaves gathered from the field site. Because the reactivity of condensed tannins varies by species, the results should only be used as an index of relative treatment responses and not as 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 least significant difference (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. The bags were strung on monofilament line in random order and suspended in the East Branch of the Maple River on 1 November 2003. The 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, MI, 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 · liter⁻¹, a mean ammonia concentration of 23.4 μg N · liter⁻¹, and a mean soluble reactive phosphorus concentration of 2.2 μg P · liter⁻¹ at the study site (J. A. Teeri, unpublished data). The mean stream width in the study reach was 5 m, and the mean depth was 0.5 m. The leaf bags were collected after 14 days in the stream and frozen for transport to Loyola University, Chicago, IL.

Microbial community size. The living biomass of the fungal communities colonizing the leaves was estimated from the concentrations of ergosterol (35). Leaves were subsampled using a sterile cork borer with a 2-cm diameter. The leaf disks were placed in high-performance liquid chromatography (HPLC) grade methanol and stored in a freezer until they were analyzed. The ergosterol in the samples was extracted, partially cleaned by solid-phase extraction (35), and quantified with a Shimadzu HPLC system. The ergosterol concentrations were converted to fungal biomass by 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 disks) 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 they were analyzed. Bacterial cells attached to leaf samples were detached by ultrasonic-probe sonication for 30 s on ice. After sonication, sample aliquots were stained and filtered onto 0.2-μm Anodisc filter membranes (Whatman, Piscataway, NJ). The 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 gram (dry weight) of leaf material.

Fungal-biomass and bacterial-count data were analyzed by two-way 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. (i) DNA isolation. Genomic DNA was isolated from each leaf pack using the Ultra Clean Soil DNA Kit (MoBio Laboratories, Salina Beach, CA). This genomic DNA was used for terminal restriction fragment length polymorphism (T-RFLP) analysis and clone library analysis, as described below.

(ii) 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 the dye IRD-800 by Li-Cor (Lincoln, NE). Each 25- μ l PCR mixture contained 0.4 μ M forward primer, 4.0 μ M reverse primer, 200 μ M deoxynucleoside triphosphates (Amersham Biosciences, Piscataway, NJ), 1 \times PCR buffer (Promega, Madison, WI), 2.5 mM MgCl₂ (Promega), 1.5 units of *Taq* DNA polymerase (Promega), and 1.0 μ l of template DNA. For these samples, a 1:10 dilution of DNA extract was found to be optimal for amplification. PCRs were run in a PTC-100 DNA thermal cycler (MJ Research, Waltham, MA). The cycling parameters were as described previously (30). Two replicate PCRs 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 PCR using the primers 8F and 926R (48). Primer 926R was synthesized by Operon, and 8F was synthesized and labeled at the 5' end with the dye IRD-800 by Li-Cor. PCRs were set up as described above for fungal community analysis. The cycling parameters were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 56.4°C for 20 s, and extension at 72°C for 30 s, with a final extension step for 3 min at 72°C. Two replicate PCRs 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 Cleanup DNA Purification Kit (MoBio Laboratories) and quantified based on analysis of agarose gel band intensities using Quantity One Software (Bio-Rad, 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 the 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 terminal restriction fragments (TRFs) were determined using Quantity One Software (Bio-Rad). 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 nonmetric multidimensional scaling (MDS) using the Primer V.5 software package (Primer-E Ltd., Plymouth, United Kingdom). 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 the 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 the similarity for samples within groups is not greater than the similarity for samples between groups. The *P* value reflects the statistical significance of the *R* statistic (19).

(iii) 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 the unlabeled primers ITS1F and ITS4 (30). Bacterial 16S rRNA genes were amplified by PCR (as described above) using the 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 *Escherichia coli*. Transformed *E. coli* cells were grown overnight on LB agar plates containing 50 μ g/ml kanamycin. Randomly selected colonies were transferred to LB broth containing 50 μ g/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 primers M13F and M13R. Each plasmid that contained an appropriately sized insert was sequenced bidirectionally using primers M13F and M13R by the University of Chicago Cancer Research Center's DNA Sequencing Facility (Chicago, IL).

TABLE 1. Chemical analyses of leaves

Species	Treatment	% Leaf dry mass ^a			C/N molar ratio ^a
		Simple phenolics	Condensed tannins	Lignin	
Aspen	Ambient	6.8 (0.0)	10.3 (0.4)	17.8 (3.4)	70.8 (4.9)
	Elevated	7.9 (0.3) ^b	16.5 (2.0) ^b	21.6 (1.8)	156.1 (26.7) ^b
Maple	Ambient	8.4 (0.9)	11.0 (0.6)	16.1 (1.0)	194.9 (11.0)
	Elevated	10.9 (0.4) ^b	20.4 (2.6) ^b	13.7 (1.2)	236.8 (25.5) ^b
Willow	Ambient	6.0 (0.3)	19.5 (1.3)	15.7 (1.1)	92.5 (0.9)
	Elevated	7.3 (0.3) ^b	24.5 (2.3) ^b	14.1 (1.1)	147.3 (7.9) ^b

^a Each data point represents the mean ($n = 3$), with standard error values in parentheses.

^b Significant difference with ELEV treatment ($P < 0.001$).

The SeqMan component of the Lasergene software package (DNASTar, Inc., Madison, WI) was used to assemble a consensus sequence for each clone. 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 to calculate a distance matrix. Operational taxonomic units (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).

Nucleotide sequence accession numbers. Fungal sequences were deposited in GenBank under accession numbers GU065446 to GU065632. Bacterial sequences were deposited in GenBank under accession numbers GU568764 to GU569021.

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 the 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 the lignin content of leaves. Leaf chemistry also varied significantly between tree species (Table 1). There was a significant effect of the tree species on the 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 the lignin content of leaves ($P < 0.05$), with aspen leaves having significantly higher lignin content than maple ($P < 0.05$) and willow ($P < 0.05$) leaves. Finally, there was a significant effect of tree species ($P < 0.001$) on the C/N ratio of leaves, with maple leaves having a significantly higher C/N ratio than aspen or willow leaves ($P < 0.001$) and no significant difference in C/N ratio 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

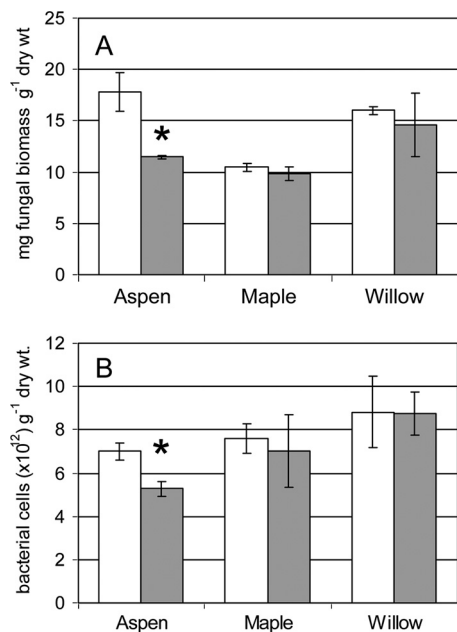


FIG. 1. Fungal biomass (A) and direct counts of bacterial cells (B) on ambient-CO₂ leaves (white bars) and elevated-CO₂ leaves (gray bars). Each bar represents the mean value (*n* = 4), and the error bars represent standard errors. Significant differences between ambient and elevated treatments (*P*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

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 versus ELEV samples across all tree species (Fig. 2A), and ANOSIM analysis showed a low *R* value (0.018) for the comparison of all AMB versus 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 versus 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

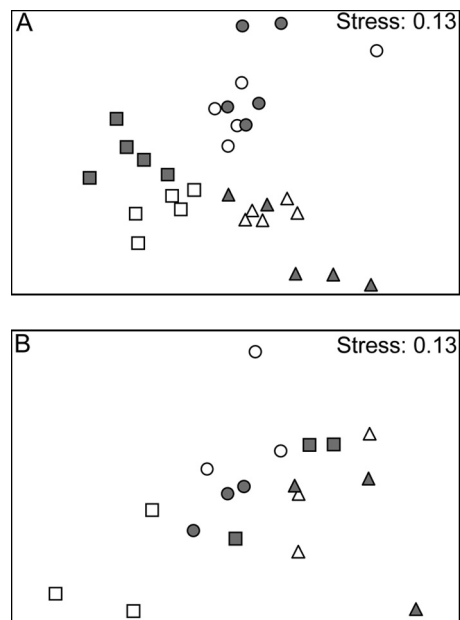


FIG. 2. Nonmetric multidimensional scaling analysis of fungal (A) and bacterial (B) T-RFLP data. Shown are aspen ambient-CO₂ leaves (open squares), aspen elevated-CO₂ leaves (gray squares), maple ambient-CO₂ leaves (open circles), maple elevated-CO₂ leaves (gray circles), willow ambient-CO₂ leaves (open triangles), and willow elevated-CO₂ leaves (gray triangles).

clear separation of AMB versus ELEV samples across all tree species (Fig. 2B), and ANOSIM analysis showed a low *R* value (0.001) for the comparison of all AMB versus 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 versus ELEV aspen samples (Table 2). In

TABLE 2. ANOSIM analyses of bacterial and fungal T-RFLP data

Comparison	Bacteria		Fungi	
	<i>R</i> statistic	<i>P</i> value	<i>R</i> statistic	<i>P</i> value
Global ^a	0.381	0.001	0.653	0.001
All ambient vs. all elevated	0.001	0.040	0.018	0.271
Aspen ambient vs. aspen elevated	0.778	0.100	0.584	0.008
Maple ambient vs. maple elevated	0.130	0.200	0.004	0.429
Willow ambient vs. willow elevated	-0.333	1.000	0.126	0.183
All aspen vs. all maple	0.207	0.050	0.715	0.001
All aspen vs. all willow	0.307	0.020	0.792	0.001
All maple vs. all willow	0.348	0.009	0.670	0.002
Aspen ambient vs. maple ambient	0.741	0.100	0.624	0.008
Aspen ambient vs. willow ambient	1.000	0.100	0.992	0.008
Maple ambient vs. willow ambient	0.111	0.400	0.664	0.008

^a The global test is based on the null hypothesis that there are no differences between any of the groups.

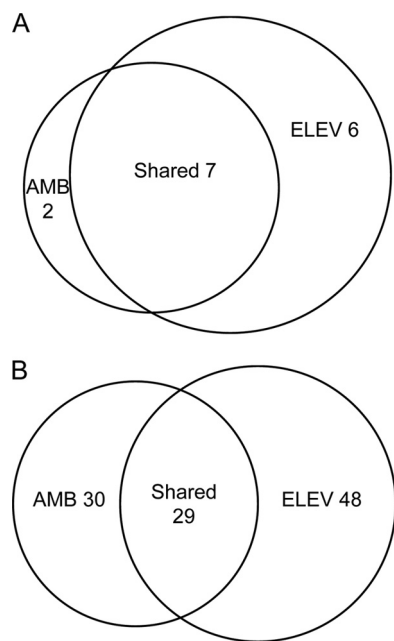


FIG. 3. Venn diagrams showing shared and unique fungal (A) and bacterial (B) OTUs (3%) based on clone libraries from AMB aspen leaves and ELEV CO₂ aspen leaves.

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 significantly high *R* values for the comparisons of aspen AMB versus maple AMB and aspen AMB versus willow AMB, respectively. Thus, there were some significant differences in bacterial community compositions 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 aspen leaves (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). Collector's 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 to 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

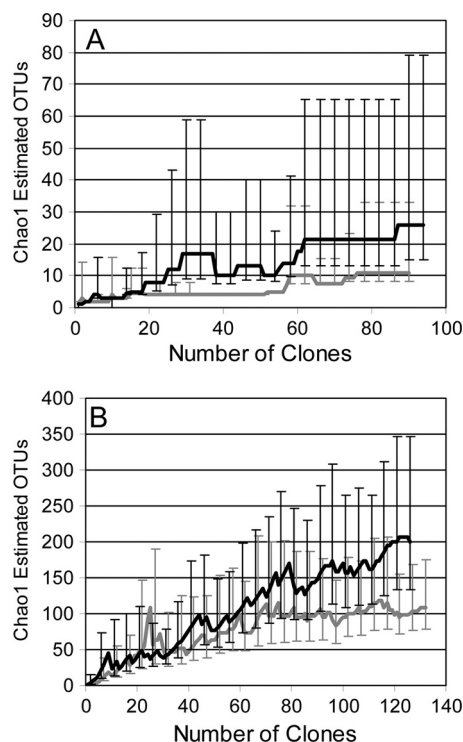


FIG. 4. Collector's curves for Chao1 estimated total numbers of fungal (A) and bacterial (B) OTUs (3% distance) based on clone libraries from ambient-CO₂ aspen leaves (gray line) and elevated-CO₂ aspen leaves (black line). The error bars represent 95% confidence intervals.

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, collector's 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 collector's curves were also fairly stable over the last 20 to 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 they were found in two OTUs, one showing 99% identity to *Aureobasidium pullulans* and one showing 90% identity to organisms from the genus *Cladosporium*. Finally,

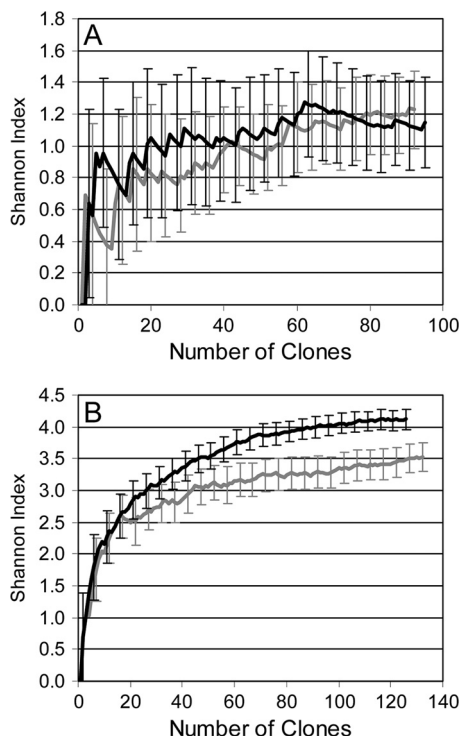


FIG. 5. Collector's curves for Shannon diversity index scores for fungal (A) and bacterial (B) clone libraries from ambient-CO₂ aspen leaves (gray line) and elevated-CO₂ aspen leaves (black line). The error bars represent 95% confidence intervals.

only 3% of the fungal ITS sequences were unique to the ELEV leaves, and they 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). Collector's 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) than on the AMB leaves (107 OTUs), but the confidence intervals indicated that these differences were not statistically significant (Fig. 4B). However, collector's curves based on the Shannon diversity index showed significantly higher bacterial diversity for the ELEV leaves than for the AMB leaves (Fig. 5B). In addition, the Shannon index collector's 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%) compared to the AMB treatment. In addition, the ELEV clone library showed a higher proportion of cytophaga-flavobacter-bacteroides (CFB) group bacteria (35% compared to 20%) and a much lower proportion of firmicutes (2% 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 the 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 *P. tremuloides* and *A. 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, an increased C/N ratio of leaves make 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 high nitrogen contents (e.g., alder and 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 ratio 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 were likely interrelated, with a decrease in one microbial group possibly influencing the 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 crane fly (*Tipula abdominalis*) larvae (70), increased mortality rates in one species of mosquito larvae (*Aedes albopictus*), and decreased development rates for several species of mosquito larvae (*Aedes triseriatus*, *Aedes aegypti*, and *Armigeres subalbatu*) (69). The fact that we observed decreased microbial colonization of ELEV aspen leaves is especially significant for our study site, since aspen (*P. tremuloides*) is the most abundant tree species in Michigan (61), and it accounts for approximately 22% of the leaf litter in our study stream (N. C. 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 than 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 the 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 to have 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 on leaf litter decomposing in streams (7, 14). However, numerous studies have identified aquatic hyphomycetes as the dominant fungal group on decomposing leaf litter in streams (for a review, see reference 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 to 6 h, and start to release conidia in as little as 6 to 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); 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 a review, see reference 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 denaturing gradient gel electrophoresis (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 be related 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 ratios 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 ratios.

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 CO₂, 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 the 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 ratio 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.

ACKNOWLEDGMENTS

Experimental treatment of trees was conducted at the Elevated CO₂ Research Facility at the University of Michigan Biological Station, where infrastructural support was provided by the U.S. DOE National Institute of Global Environmental Change (NIGEC). This research was supported by grants awarded to N.C.T. from the National Science Foundation (DEB-9903888 and DEB-0108847) and by a supplement to grant DEB-0108847 awarded to N.C.T. and J.J.K. from the National Science Foundation. Work on this project was supported by fellowships awarded to A.B. and M.W. from the Loyola University Chicago WISER and Mulcahy Scholars programs and the Loyola University Chicago Center for Urban Environmental Research and Policy. P.B.'s work on this project was made possible by participation in the Loyola NSF-REU Program in Bioinformatics (DBI-0552888). Preparation of the manuscript was supported by a Faculty Scholarship Stimulation Award granted to J.J.K. by the Loyola University Chicago College of Arts and Sciences.

We thank the Department of Civil and Environmental Engineering at Northwestern University for use of the Li-Cor DNA sequencer, Martin Berg for assistance with statistical analysis, Michael Grant of the University of Michigan Biological Station for chemical analysis of leaves, and Anna Taber for assistance in the early stages of this study.

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