

# Chemical changes to leaf litter from trees grown under elevated CO<sub>2</sub> and the implications for microbial utilization in a stream ecosystem

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**Abstract:** Chemical alterations to leaf litter associated with growth under elevated CO<sub>2</sub> may impact aquatic ecosystems that rely on terrestrial leaf litter as a carbon source. This study examined how elevated CO<sub>2</sub> altered the chemistry and subsequent response of stream microorganisms growing on the leaf litter of three riparian tree species. Quaking aspen (*Populus tremuloides*), white willow (*Salix alba*), and sugar maple (*Acer saccharum*) were grown under ambient (360 parts per million) and elevated (720 parts per million) CO<sub>2</sub> for an entire growing season and senesced leaf litter was incubated in a stream for 80 days. Elevated-CO<sub>2</sub> effects on the chemistry of senesced litter were species-specific. Aspen leaves contained higher concentrations of lignin, maple leaves contained higher concentrations of soluble phenolic compounds, and willow leaves contained higher concentrations of carbohydrate-bound condensed tannins. Initially higher concentrations of soluble phenolic compounds in maple leaves were rapidly leached in stream water. However, higher concentrations of carbohydrate-bound tannins in elevated-CO<sub>2</sub>-grown willow leaves persisted and were correlated with reduced phenol oxidase activities of attached microbiota. Overall, altered leaf chemistry associated with growth under elevated CO<sub>2</sub> did not strongly suppress microbial activity during stream incubation. In cases where there was evidence of suppression, it was largely species-specific.

**Résumé :** L'altération chimique de la litière de feuilles dans des conditions de croissance sous des concentrations élevées de CO<sub>2</sub> peut affecter les écosystèmes aquatiques qui dépendent de l'apport de litière de feuilles terrestres comme d'une de leurs sources de carbone. Notre étude examine comment un accroissement du CO<sub>2</sub> modifie les réactions chimiques et, par conséquent, les réactions des microorganismes d'eau courante qui se développent sur la litière de feuilles de trois espèces d'arbres riverains. Nous avons cultivé des peupliers faux-trembles (*Populus tremuloides*), des saules blancs (*Salix alba*) et des érables à sucre (*Acer saccharum*) dans des conditions de CO<sub>2</sub> ambiant (360 parties par million) et dans des conditions élevées (720 parties par million) durant une saison entière de croissance; nous avons ensuite incubé la litière parvenue à l'état sénescence dans un cours d'eau pendant 80 jours. Les effets de l'augmentation du CO<sub>2</sub> sur la chimie de la litière sénescence varient d'une espèce à l'autre. Les feuilles de tremble contiennent plus de lignine, celles d'érable des concentrations plus élevées de composés phénoliques solubles et celles de saule des teneurs plus grandes de tanins condensés liés aux hydrates de carbone. Les concentrations initiales plus fortes de composés phénoliques solubles dans les feuilles d'érable sont vite lessivées dans l'eau de cours d'eau. Cependant, les concentrations plus grandes de tanins liés aux hydrates de carbone dans les feuilles de saules cultivés dans des conditions élevées de CO<sub>2</sub> se maintiennent et sont en corrélation avec l'activité réduite de la phénol oxydase dans les microorganismes qui leur sont associés. En général, la modification de la chimie des feuilles dans les conditions de croissance sous des concentrations élevées de CO<sub>2</sub> ne diminue pas de façon importante l'activité microbienne durant l'incubation dans un cours d'eau. Dans les cas où il y a des indices d'une diminution, cette diminution varie en grande partie d'une espèce à l'autre.

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## Introduction

Rising levels of atmospheric CO<sub>2</sub> will potentially have far-reaching impacts on both terrestrial and aquatic ecosystems. A doubling in atmospheric CO<sub>2</sub> by the end of this century (Houghton et al. 2001) may lead to increased rates of carbon fixation by vascular C<sub>3</sub> plants (e.g., Gill et al. 2002). This decrease in carbon limitation will likely be accompanied by a shift to limitation by other nutrients such as nitrogen in many ecosystems. Under these conditions plants may allocate more carbon to secondary compounds like tannins, while nitrogen-rich compounds become more dilute (e.g., Wetzel and Grace 1983; Lindroth et al. 1993; Strain and Bazaaz 1983).

In addition to potentially having negative effects on terrestrial herbivores (e.g., Lindroth et al. 1993; Lindroth and Kinney 1998) and the decomposition of leaf litter in terrestrial environments (e.g., Cotrufo and Ineson 1996; De Angelis et al. 2000), elevated-CO<sub>2</sub>-induced alterations in the chemistry of deciduous leaves may also negatively impact many aquatic ecosystems. Headwater streams as well as larger rivers depend on subsidies of allochthonous organic matter for up to 99% of the annual carbon budget (Minshall 1967; Fisher and Likens 1973; Wetzel 2001). Therefore, alterations in the chemistry of terrestrial leaf litter that result in increased resistance to decomposition will potentially influence the chemical composition of and capacities for enzymatic hydrolysis and oxidation by microbiota in these ecosystems. This hypothesis has been supported in several short-term studies where quaking aspen (*Populus tremuloides* Michaux) was grown under both ambient (360 parts per million (ppm)) and elevated (720 ppm) concentrations of atmospheric CO<sub>2</sub>, the naturally senesced leaf litter being subsequently incubated in a stream (Tuchman et al. 2002, 2003b; Rier et al. 2002) or a laboratory microcosm (Wetzel and Tuchman 2004). For example, Tuchman et al. (2002) demonstrated that elevated-CO<sub>2</sub>-grown quaking aspen leaves placed in a stream for 14 days had less than half of the biomass-specific bacterial productivity of those incubated under ambient CO<sub>2</sub>. Rier et al. (2002), also working with quaking aspen leaves, found negative effects on the microbial community in the first 12 days of incubation. However, between 12 and 30 days, it appeared that a combination of leaching of soluble phenols and colonization by aquatic fungi, which increased leaf nitrogen, might compensate for any initial chemical differences between elevated- and ambient-CO<sub>2</sub>-grown leaves.

Although the above studies indicate that an atmosphere enriched with CO<sub>2</sub> could indirectly affect leaf-litter quality and subsequent microbial utilization, at least initially, a need exists to evaluate the ubiquity of such effects in stream ecosystems. Litter from several tree species needs to be assessed, the effects on stream microorganisms need to be determined over longer periods of time (over 30 days), and potential compensatory mechanisms such as those shown by Rier et al. (2002) need to be investigated further.

The objective of the current study was to examine how elevated CO<sub>2</sub> altered the chemistry and the subsequent response of stream microorganisms to the leaf litter of three tree species common to the riparian zones of northern Michigan streams. The three species, chosen on the basis of de-

composition rates reported in the literature (sensu Petersen and Cummins 1974), were quaking aspen (slow decomposition rate), white willow (*Salix alba* L.) (moderate decomposition rate), and sugar maple (*Acer saccharum*) (fast decomposition rate). Initial chemical differences between the leaf litter of ambient- and elevated-CO<sub>2</sub>-grown trees were assessed, and changes in bacterial biomass, fungal biomass, phenol oxidase activity (POA), and leaf chemistry were followed throughout an 80-day in-stream incubation.

## Methods

### Tree growth under elevated CO<sub>2</sub>

Quaking aspen, white willow, and sugar maple were grown at The University of Michigan Biological Station in northern Lower Michigan. Although the white willow is not native to northern Michigan, it is found in the riparian zone of the stream where the in-situ part of this experiment was run. Sixteen 6-year-old quaking aspen clones, twenty 2-year-old white willow clones, and twenty 2-year-old sugar maple siblings (collected under one parent tree) were grown in open-bottom root boxes containing a soil homogenate of 80% native Kalkaska rubicon sand and 20% topsoil. This mixture provided nutrient levels that were comparable to those of soils in this region (Zak and Pregitzer 1990). Trees were enclosed in 1-m<sup>3</sup> open-top chambers, air being circulated through each with a blower fan (see Curtis and Teeri 1992). Half of the chambers were maintained at ambient atmospheric CO<sub>2</sub> concentrations (360 ppm), while half were maintained at elevated levels (720 ppm) by dispensing 100% CO<sub>2</sub> into the blower fans. Elevated CO<sub>2</sub> concentrations were maintained by continuously monitoring CO<sub>2</sub> concentrations in each elevated-level and one ambient-level chamber with an infrared gas analyzer (LICOR<sup>®</sup> model LI-6252, Lincoln, Nebraska, USA) and then periodically adjusting the flow of 100% CO<sub>2</sub> into each chamber. Treatments were maintained through the entire growing season, naturally senesced leaf litter being collected at the end of the season.

### In-stream incubation

Leaf litter was incubated in a third-order section of the East Branch Maple River (45°34.505'N, 84°44.706'W) near Pellston, Michigan. The East Branch Maple River originates from Douglas Lake approximately 3 km northeast of the study site, where it flows through undeveloped wetlands and northern hardwood forests. Nitrogen and phosphorus concentrations were low in this section of stream, with a mean nitrate concentration of 13 µg N·L<sup>-1</sup>, a mean ammonia concentration of 25 µg N·L<sup>-1</sup>, and a mean soluble reactive phosphorus concentration of 2.2 µg P·L<sup>-1</sup> (R. Vande Kopple, University of Michigan Biological Station Stream Research Facility, 9008 Biological Road, Pellston, MI 49769, USA, unpublished data). Mean stream width in the study reach was 5 m and mean depth was 0.5 m.

Incubation was conducted between February and April 2002. Ambient- and elevated-CO<sub>2</sub>-grown leaves of each species were placed individually in 1.4-mm-mesh bags (*n* = 120 bags) to discourage macroinvertebrate colonization, and suspended in the East Branch Maple River at a current velocity of ~20 cm·s<sup>-1</sup>. Six ambient- and six elevated-CO<sub>2</sub>-grown

leaves of each species were retrieved on days 10, 20, 40, and 80. Stream temperature was monitored with an Onset Stow-Away<sup>®</sup> XTI temperature recorder (Onset Computer, Bourne, Massachusetts, USA) set to take a reading once every hour.

Leaves collected from the stream were placed in individual zipper bags filled with stream water and immediately transported to the laboratory. Leaves were removed from the mesh bags, gently rinsed in stream water to remove silt and fine particulate organic matter, and then subsampled with a 7.2 mm diameter cork borer to determine bacterial productivity (composite sample of five disks), bacterial biomass (composite sample of five disks), fungal biomass (composite sample of five disks), and POA (one 31 mm diameter leaf disk). The remaining leaves were assayed for total phenols (TPh), soluble condensed tannins (CT), carbohydrate-bound condensed tannins (BT), the ratio of organic carbon to organic nitrogen (C:N), and lignin.

### Microbial assays

Bacterial productivity was determined by measuring the incorporation of <sup>3</sup>H-leucine into protein. Leaf disks were first briefly sonicated for <30 s in 10 mL of sterile filtered (0.2 µm pore size) stream water to remove bacteria (Thomaz and Wetzel 1995). The leaf disks were removed and the remaining sample was incubated for 1 h at 4 °C after the addition of 10 µL of <sup>3</sup>H-leucine (99.9 Ci·mmol<sup>-1</sup>; 1 Ci = 37 Gbc). Bacterial productivity was estimated after accounting for quenching and subtracting the activity of a killed control (Kirchman 1993).

Bacterial biomass was determined on the leaf disks that were preserved in a mixture of 3.7% formaldehyde and 0.1 mol·L<sup>-1</sup> tetrasodium pyrophosphate (Velji and Albright 1986). Bacterial cells were removed from the leaf disks by sonicating for 30 s on ice. Cells were stained with 4',6'-diamidino-2-phenylindole and enumerated using epifluorescent microscopy. Cell biovolumes were determined using geometric shapes (Psenner 1993; Wetzel and Likens 2000) and total carbon was estimated by multiplying biovolumes by 5.6 × 10<sup>-13</sup> g C·µm<sup>-3</sup> (Bratbak 1985).

Fungal biomass was estimated from total ergosterol content determined with high performance liquid chromatography (HPLC) analyses (Newell and Fallon 1991; Newell 1995; Wetzel and Likens 2000). Briefly, disks were placed in 10 mL of methanol (HPLC grade) in reflux flasks and refluxed at 80 °C for 30 min in a block heater. Following addition of 5 mL of ultrapure water and 5 mL of pentane (HPLC grade) and mixing by inversion 30 times, the water was removed from the nonpolar pentane fractions. The extraction was repeated 2 additional times. The pentane fractions were evaporated at 30 °C and the lipids were redissolved in 2.0 mL of methanol (HPLC grade) with sonication for 5–10 min. Following filtration, the samples were analyzed by HPLC calibrated against pure standards. Fungal carbon was calculated by multiplying ergosterol concentrations by a ratio of 1 mg of fungal dry mass per 5.5 µg of ergosterol (Gessner and Chauvet 1993).

POA was measured on days 20, 40, and 80. A 3.1-cm diameter disk from each leaf was pulverized in a Dentsply Rinn<sup>®</sup> model C32003A Wiggle Bug (Dentsply Rinn, Elgin, Illinois, USA) and suspended in 3 mL of 50 mmol·L<sup>-1</sup> acetate

buffer, pH 5.0. This suspension was divided into two 1.5-mL subsamples in glass culture tubes. One subsample received 0.5 mL of a solution of L-3,4-dihydroxyphenylalanine (L-DOPA) in acetate buffer to yield a final concentration of 5 mmol·L<sup>-1</sup> L-DOPA (Sinsabaugh et al. 1994). The other subsample was used as a blank and received an additional 0.5 mL of acetate buffer. Tubes were capped and incubated at 4 °C for 1 h on a shaker table. Following the incubation period, the tubes were centrifuged and the supernatant was removed and the absorbance was read at 460 nm on a Spectronic<sup>®</sup> Genesys<sup>™</sup> 2 spectrophotometer (Thermo Electron Corporation, Waltham, Massachusetts, USA). POA was calculated in micromoles of substrate converted per hour per gram of leaf dry mass.

### Leaf chemistry

Leaf material from days 0 (not incubated in the stream), 20, 40, and 80 was assayed for TPh, CT, BT, and C:N. TPh concentrations were determined using the Folin–Denis assay (Swain and Goldstein 1964) after extraction of 20 mg of sample in 1.5 mL of 70% acetone for 90 min in an ultrasonic water bath maintained at 0 °C. Tannic acid was used as a standard in this assay. Phenol concentrations are therefore based on tannic acid equivalence and are expressed as percent dry mass. CT concentrations were determined from 70% acetone extracts using the acid butanol method (Porter et al. 1986). Concentrations are based on leucocyanidin equivalence and are expressed as percent dry mass.

BT concentrations were determined using a method modified from Terrill et al. (1992). Leaf material that was left behind after extraction with 70% acetone was dried and reweighed. Protein was removed from the material by extracting with a mixture of 1.25 mL of H<sub>2</sub>O and 0.75 mL of phenol at 95 °C for 2 h. The samples were centrifuged and the phenol–H<sub>2</sub>O mixture was removed. The material was then washed 3 times with ether to remove excess phenol. After drying, condensed tannin concentrations were determined using the acid butanol method. Condensed tannin purified from ambient-CO<sub>2</sub>-grown quaking aspen leaves (for the purification procedure see Terrill et al. 1992) was used as a standard in this assay. Serial dilutions of this purified standard dissolved in 70% acetone were dried in a stream of helium before undergoing the acid butanol hydrolysis.

C:N was determined on a Perkin Elmer<sup>®</sup> 2400 elemental analyzer (Perkin Elmer, Wellesley, Massachusetts). Lignin concentrations were determined on initial samples (day 0) using an acetyl bromide method according to Iiyama and Wallis (1990). Ten milligrams of ground material was extracted in 65 °C water for 1 h followed by filtering onto glass-fiber filters and rinsing with water, ethanol, acetone, and diethyl ether. This step was followed by heating overnight at 50 °C and extracting in a 25% solution of acetyl bromide in acetone at 50 °C. Absorbance of extracts was determined at 280 nm on a Spectronic<sup>®</sup> Genesys<sup>™</sup> 2 spectrophotometer (Thermo Electron Corporation).

### Data analysis

All statistical analyses were performed using Systat<sup>®</sup> version 10 (Systat Software Inc. 2000). Since BT, CT, TPh, and lignin concentrations are expressed as a percentage of leaf

**Table 1.** Probabilities ( $p$ ) obtained from running two-way ANOVAs on initial chemical parameters for leaf litter produced by three riparian tree species (quaking aspen (*Populus tremuloides*), sugar maple (*Acer saccharum*), and white willow (*Salix alba*)) grown at both ambient (360 parts per million (ppm)) and elevated (720 ppm) concentrations of atmospheric CO<sub>2</sub>.

	CO <sub>2</sub> treatment	Species	CO <sub>2</sub> treatment × species
Total soluble phenols	<0.001	<0.001	<0.001
Soluble condensed tannins	0.162	<0.001	0.759
Carbohydrate-bound condensed tannins	0.038	<0.001	0.036
Lignin	0.473	0.181	0.032
C:N	0.019	<0.001	0.118

dry mass, these data were first arcsine ( $x^{0.5}$ ) transformed to improve normality. Estimates of C:N, bacterial carbon, fungal carbon, and POA were  $\log(x + 1)$  transformed to improve normality and normalize variances. A two-way analysis of variance (ANOVA) with species and CO<sub>2</sub> treatment as factors were run on each initial chemical parameter. Three-way ANOVAs with time, species, and CO<sub>2</sub> treatment as factors were used to compare chemistry and microbial data throughout the 80-day in-situ incubation. Correlation analysis was also run between POA and BT, CT, and TPh concentrations in leaf litter collected at all stages of the incubation. Probabilities obtained in the correlation analysis were Bonferroni-corrected to limit overall experimental error rate.

## Results

### Initial chemical differences

Elevated-CO<sub>2</sub>-induced changes in the chemistry of senesced leaf litter were species-specific. There was a significant species × CO<sub>2</sub> interaction ( $p = 0.001$ ; Table 1) with respect to TPh concentration, which indicates that the production of simple phenols, hydrolysable tannins, and condensed tannins under elevated CO<sub>2</sub> was largely species-specific. Maple leaf litter had the highest TPh concentration, while aspen had the lowest (Fig. 1a). Within individual species, concentrations were 36% higher in elevated-CO<sub>2</sub>-grown maple than in ambient-CO<sub>2</sub>-grown maple, a difference of only 12% and 8% being observed between the leaf litter of ambient- and elevated-CO<sub>2</sub>-grown aspen and willow, respectively. Although mean CT concentrations were slightly higher in all three species in the elevated-CO<sub>2</sub> treatments (Fig. 1b), species was the only statistically significant factor ( $p < 0.001$ ; Table 1). The BT concentration was highest in aspen and lowest in maple (Fig. 1c). The effect of elevated CO<sub>2</sub> on BT was species-specific, with a significant CO<sub>2</sub> × species interaction ( $p = 0.036$ ; Table 1). Concentrations were higher in willow leaves grown under elevated CO<sub>2</sub> than in those grown under ambient CO<sub>2</sub>, with leaves grown at elevated CO<sub>2</sub> having nearly twice the BT concentration (Fig. 1c). Elevated CO<sub>2</sub> did not produce substantial differences in BT concentration in maple or aspen leaf litter. The response of lignin concentrations to elevated CO<sub>2</sub> was also species-specific, with a significant CO<sub>2</sub> × species interaction ( $p = 0.032$ ; Table 1). Elevated-CO<sub>2</sub>-grown aspen leaf litter was 15% higher in lignin than that grown under ambient CO<sub>2</sub> (Fig. 1d). Species and CO<sub>2</sub> treatment independently affected C:N, with significant species ( $p < 0.001$ ) and CO<sub>2</sub> treatment

( $p = 0.019$ ) effects with no interaction ( $p = 0.118$ ). C:N was highest in maple followed by willow and then aspen (Fig. 1e). Estimates of C:N for maple grown under elevated CO<sub>2</sub> were 25% higher than those for maple grown under ambient CO<sub>2</sub>. Estimates for willow and aspen litter were approximately 10% higher for leaves produced under elevated CO<sub>2</sub>.

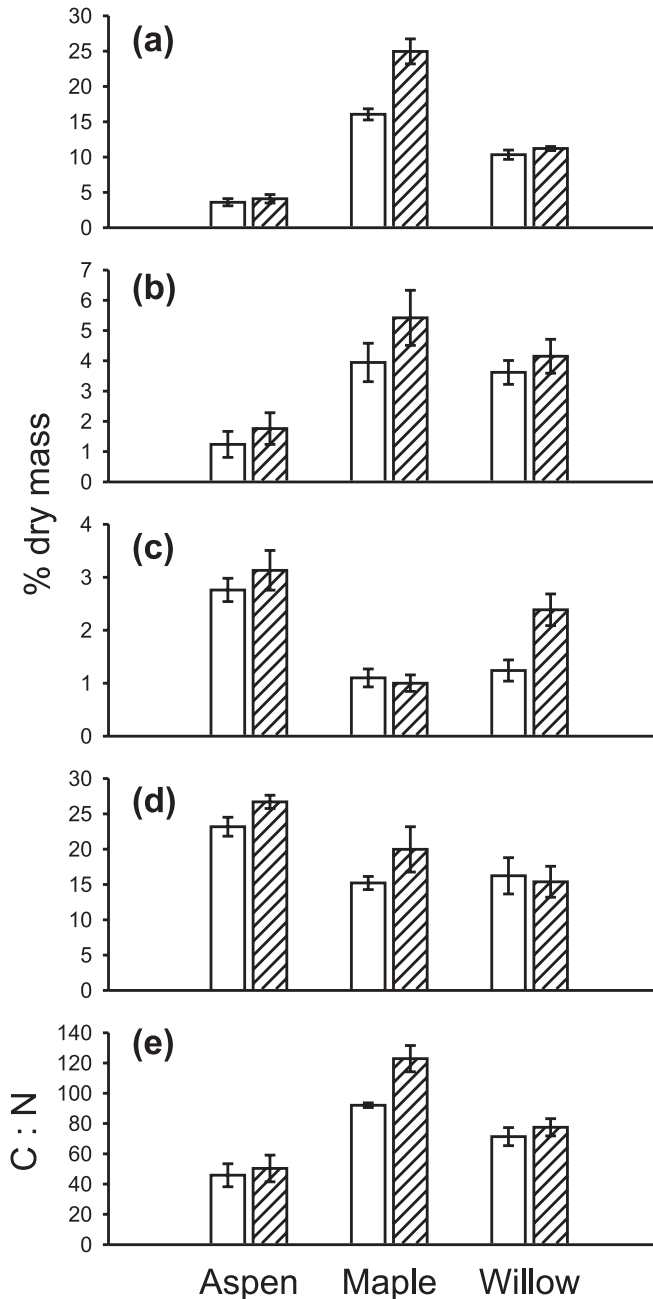
### In-situ incubation

Mean daily stream temperatures ranged from -0.1 to 13.1 °C. The average stream temperature for the entire incubation was 4.1 °C. Leaf chemistry changed in all species throughout the incubation period, and in some cases, differences between ambient- and elevated-CO<sub>2</sub>-grown leaves became either more apparent or less apparent after incubation in the stream (Fig. 2). Changes in BT through time were largely species-dependent, with a significant time × species interaction ( $p < 0.001$ ; Table 2). BT in aspen leaves decreased slightly throughout the experiment (Fig. 2a), while BT in maple increased throughout the first half of the incubation and then leveled off (Fig. 2b). BT in willow increased sharply between days 0 and 20 and then declined throughout the remainder of the incubation (Fig. 2c). CO<sub>2</sub> treatment was also a significant factor ( $p = 0.020$ ) during the incubation. CO<sub>2</sub>-treatment effects were most apparent in willow leaf litter (Fig. 2c). Throughout the experiment, with the exception of day 80, BT was highest in elevated-CO<sub>2</sub>-grown willow. The greatest difference was on day 20, when elevated-CO<sub>2</sub>-grown leaves had almost twice the BT concentration of ambient-CO<sub>2</sub>-grown leaves.

CT declined asymptotically in all three species throughout the experiment, although the magnitude of this decline varied among species (Figs. 2d–2f). Overall, there was a significant time × species interaction ( $p < 0.001$ ; Table 2). Although no initial CO<sub>2</sub> effects on CT were observed for these three species, a slight but significant CO<sub>2</sub> effect ( $p = 0.020$ ) was observed during the incubation (Fig. 2e). Similarly, TPh declined in all three species over the course of the experiment (Figs. 2g–2i), with a significant time × species interaction ( $p < 0.001$ ; Table 2). There was also a significant effect ( $p = 0.002$ ) of elevated-CO<sub>2</sub>-grown leaves on TPh during the incubation.

C:N declined nearly linearly from initial values throughout the incubation (Figs. 2j–2l). A significant time × species interaction ( $p < 0.038$ ; Table 2) was observed along with a highly significant CO<sub>2</sub> effect ( $p < 0.001$ ). Differences in C:N between the leaf litter of ambient- and elevated-CO<sub>2</sub>-grown

**Fig. 1.** Initial chemistry values (mean  $\pm$  1 standard error) including total soluble phenols (a), soluble condensed tannins (b), carbohydrate-bound condensed tannins (c), lignin (d), and the ratio of organic carbon to nitrogen, C:N (e), of naturally senesced leaf litter collected from three riparian tree species (quaking aspen (*Populus tremuloides*), sugar maple (*Acer saccharum*), and white willow (*Salix alba*) grown at both ambient (360 parts per million (ppm); open bars) and elevated (720 ppm; hatched bars) concentrations of atmospheric CO<sub>2</sub>.



trees were most apparent initially in maple (Fig. 2k) and at the midpoint of the incubation in willow (Fig. 2l).

Bacterial biomass developing on leaf litter of all three species increased through the first 40 days of in-situ incubation and then plateaued (Figs. 3a–3c). All three variables

(time, species, and CO<sub>2</sub> treatment) independently produced significant (Table 2) effects on bacterial biomass. Mean bacterial biomass was consistently lower on tissues that had been grown at elevated CO<sub>2</sub> in all species, but more so on the aspen litter (Fig. 3a). The productivity of attached bacteria on leaves of all species increased throughout the incubation (Figs. 3d–3f), producing a significant time effect ( $p < 0.001$ ; Table 2). However, there were no species or CO<sub>2</sub> treatment effects (Table 2). Fungal biomass remained low on all species throughout the first 40 days of the experiment, and then more than doubled during the final 40 days (Figs. 3g–3i), producing a significant time  $\times$  species interaction ( $p = 0.018$ ; Table 2). However, whether leaves were grown at ambient or elevated CO<sub>2</sub> had no significant effect on fungal biomass ( $p = 0.786$ ).

POA was generally higher on the aspen leaves than on leaves of the other two species (Figs. 3j–3l), and increased throughout the incubation (Fig. 3j). POA on maple leaves declined slightly between days 20 and 40 and then increased slightly between days 40 and 80 (Fig. 3k). POA on willow leaves remained constant between days 20 and 40 and then increased between days 40 and 80 (Fig. 3l). There were significant time  $\times$  species ( $p < 0.001$ ) and CO<sub>2</sub>  $\times$  time ( $p = 0.027$ ) interactions with respect to POA (Table 2). The effect of elevated CO<sub>2</sub> was most apparent in willow, where ambient-CO<sub>2</sub>-grown leaves displayed higher POA throughout the incubation (Fig. 3l). In addition, POA on willow leaf litter was negatively correlated with BT ( $r = -0.517$ ,  $p < 0.001$ ), CT ( $r = -0.349$ ,  $p = 0.001$ ), and TPh ( $r = -0.316$ ,  $p = 0.003$ ).

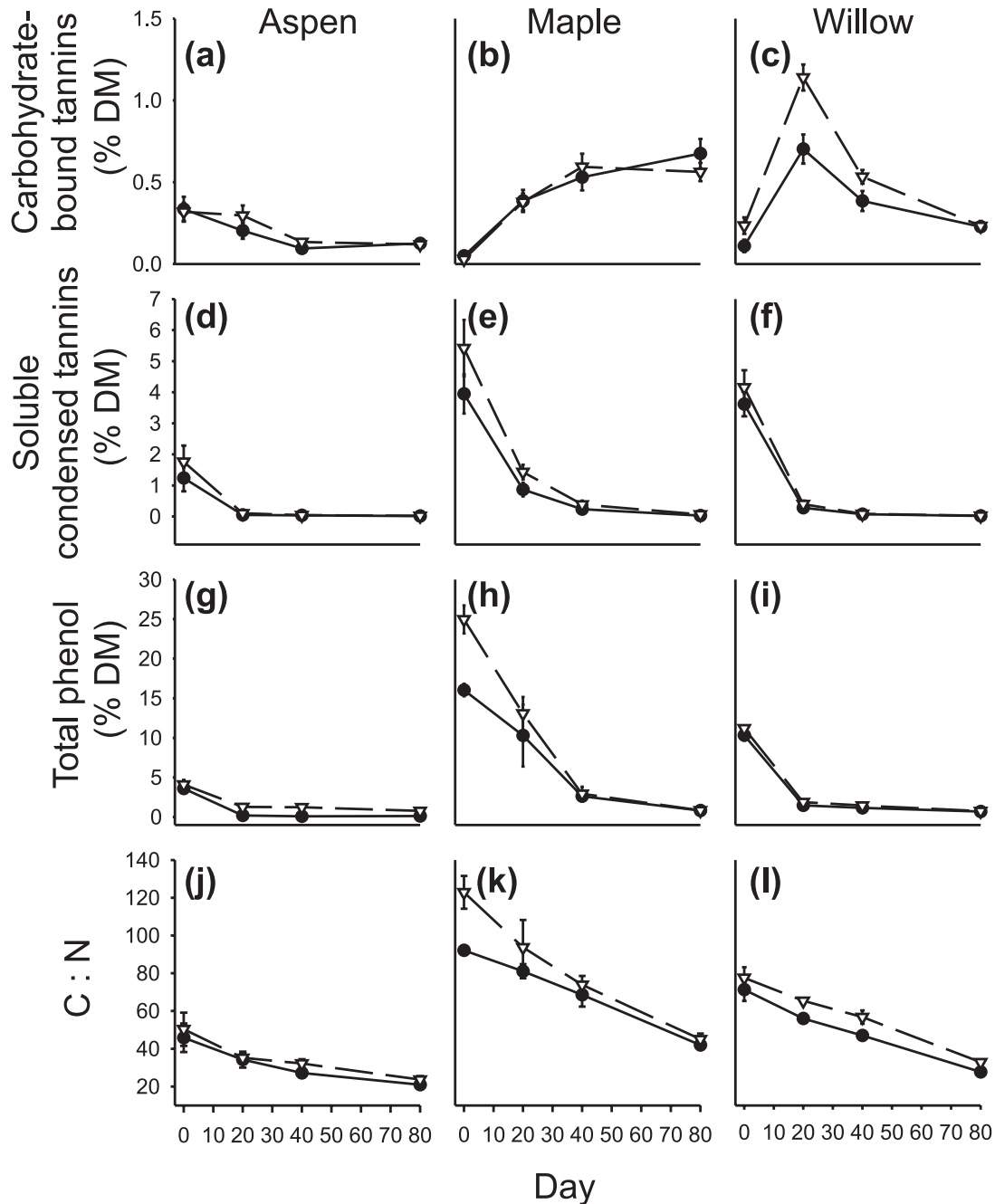
## Discussion

### Litter chemistry

Alterations of leaf-litter chemistry resulting from growth under elevated CO<sub>2</sub> was species-specific, but in all species favored production of carbon-rich compounds such as lignin, tannins, and other phenols, which were maintained after leaf senescence. Lindroth et al. (1993) demonstrated a similar species-specific response to elevated CO<sub>2</sub>, with sugar maple producing more phenolic defense compounds and quaking aspen producing more structural carbohydrates. King et al. (2001a, 2001b) found similar differences between sugar maple and quaking aspen and speculated that since quaking aspen is a fast-growing early-successional species, it will allocate more carbon to growth, whereas sugar maple, a slow-growing late-successional species, will invest more carbon into secondary metabolites.

Since growth under elevated CO<sub>2</sub> caused an increase in BT but not a substantial increase in CT in the willow leaf litter, future studies into the effects of elevated CO<sub>2</sub> on plant phenolic compounds should assay for both types of condensed tannins because both show potentially important ecological responses. Most studies into the effects of elevated CO<sub>2</sub> on leaf-litter decomposition only measure the fraction of condensed tannins that is soluble in either acetone or methanol (e.g., King et al. 2001a, 2001b; Veteli et al. 2002). Even though soluble phenols may have the potential to inhibit leaf-litter decomposition in aquatic systems (Stout 1989), concentrations generally are not highly correlated

**Fig. 2.** Leaf litter chemistry values (mean  $\pm$  1 standard error) including total soluble phenols (*a-c*), soluble condensed tannins (*d-f*), carbohydrate-bound condensed tannins (*g-i*), and C:N (*j-l*) throughout an 80-day in-stream incubation of three common riparian tree species (quaking aspen, sugar maple, and white willow) grown at both ambient (360 parts per million (ppm); solid lines) and elevated (720 ppm; broken lines) concentrations of atmospheric CO<sub>2</sub> (DM is dry mass).



with decomposition rates (Ostrofsky 1993; Gessner and Chauvet 1994; Campbell and Fuchshuber 1995). The lack of correlation between soluble phenol concentrations and decomposition rates may be partially due to leaching. For example, Benstead (1996), working in a tropical stream, showed that soluble phenols are leached out of leaf litter shortly after immersion. Rier et al. (2002), working with ambient- and elevated-CO<sub>2</sub>-grown aspen leaves demonstrated that this fraction is rapidly leached, after immersion in water for only 24–48 h. In the current study, BT persisted

in both maple and willow throughout the 80-day in-stream incubation. The difference between ambient- and elevated-CO<sub>2</sub>-produced willow leaf litter persisted throughout much of the incubation and may help to explain the persistence of a small difference in C:N between the two treatments.

#### Microbial response

Despite detectable changes in leaf-litter chemistry following growth under elevated CO<sub>2</sub>, differences in rates of colonization by fungi and bacteria in the stream incubations

**Table 2.** Probabilities ( $p$ ) obtained from running three-way ANOVAs on leaf chemistry and microbial parameters for leaf litter produced by three riparian tree species (quaking aspen, sugar maple, and white willow) grown at both ambient (360 parts per million (ppm) and elevated (720 ppm) concentrations of atmospheric CO<sub>2</sub> during an 80-day in-stream incubation.

	CO <sub>2</sub> treatment	Species	Time	Time × species	CO <sub>2</sub> treatment × time	CO <sub>2</sub> treatment × species	CO <sub>2</sub> treatment × time × species
<b>Leaf chemistry</b>							
Total soluble phenols	0.011	<0.001	<0.001	<0.001	0.077	0.284	0.136
Soluble condensed tannins	0.002	<0.001	<0.001	<0.001	0.156	0.097	0.922
Carbohydrate-bound condensed tannins	0.020	<0.001	<0.001	<0.001	0.149	0.143	0.467
C:N	0.001	<0.001	<0.001	0.038	0.883	0.569	0.839
<b>Microbial parameter</b>							
Bacterial biomass	0.013	<0.001	<0.001	0.967	0.172	0.515	0.823
Bacterial productivity	0.609	0.750	<0.001	0.594	0.790	0.693	0.944
Fungal biomass	0.876	0.458	<0.001	0.018	0.564	0.270	0.221
Phenol oxidase activity	0.786	<0.001	<0.001	<0.001	0.027	0.105	0.518

were small. Initially, the largest chemical difference was a 36% increase in TPh among maple leaves grown at elevated CO<sub>2</sub>. The difference may have produced the observed differences in bacterial biomass in maple after the first 20 days of incubation. However, this difference was no longer apparent after 20 days and likely resulted from leaching of soluble phenols from the leaves within several days of immersion.

The greater persistence of higher bacterial biomass on ambient-CO<sub>2</sub>-produced aspen litter than on elevated-CO<sub>2</sub>-produced aspen litter likely resulted from changes induced by elevated CO<sub>2</sub> that were not associated with soluble phenols, which appeared to be readily leached. It is possible that the initially higher lignin content of the elevated-CO<sub>2</sub>-produced aspen litter might have persisted throughout the incubation, preventing bacteria from utilizing the more labile carbon substrates associated with the leaf matrix.

Two methodological limitations may have contributed to an inability to detect treatment effects on bacterial productivity and fungal biomass. Because the bacteria were first removed from the surface of the leaf litter using sonication, it is likely that bacterial productivity in suspension from that on the surface of the leaf litter. It is also possible that the physical disruption associated with sonication metabolically altered the bacteria, although this limitation has been found to be minor (cf. Thomaz and Wetzel 1995). Another possible limitation is the stream temperature at the beginning of the experiment. This experiment was performed during the winter, when the temperature of the stream often approached freezing. It is possible that bacterial productivity and fungal development during the first half of the experiment were more limited by temperature than by the presence or absence of various chemical substrates. This might help to explain the overall low fungal biomass throughout this experiment relative to other studies (e.g., Gessner and Chauvet 1994).

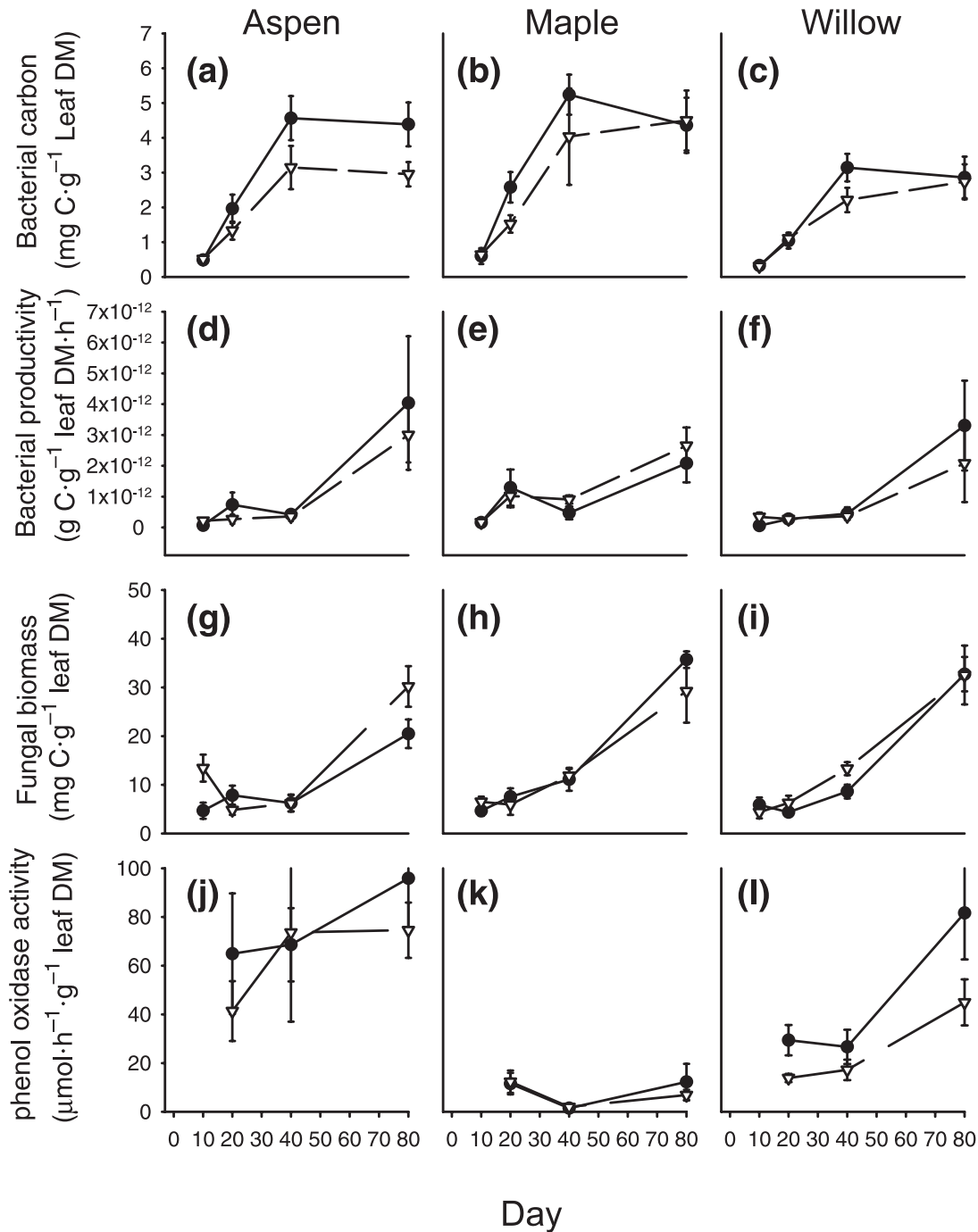
The observed differences in POA between the litter of ambient- and elevated-CO<sub>2</sub>-grown willows may have resulted from the higher concentrations of polyphenolic compounds in the elevated-CO<sub>2</sub>-grown litter. We originally hypothesized that higher concentrations of polyphenolic compounds — associated with growth under elevated CO<sub>2</sub> — would stimulate the production of phenol oxidases by the microbial com-

munity in order to utilize these compounds. However, the opposite trend was observed, with highly significant negative correlations between BT, CT, and TPh and POA. It is possible that POA was inhibited by condensed tannins that were bound to carbohydrates, CT that were in the process of leaching from the litter, or other soluble phenolic compounds. Enzyme inhibition by these compounds has been known for many decades (e.g., Haslam 1988) and it has been widely observed that tannins and other phenolic compounds complex many different membrane-bound and extracellular enzymes by noncompetitive inhibition (e.g., Stewart and Wetzel 1982; Wetzel 1991, 1993).

A number of terrestrial studies have not shown appreciable differences in leaf-litter decomposition among plants grown at ambient and elevated CO<sub>2</sub>. Norby et al. (2001), using meta-analysis to examine a large array of published and unpublished data, demonstrated that although growth under elevated CO<sub>2</sub> does affect leaf-litter chemistry, these effects do not result in slower rates of decomposition. In addition, in studies with quaking aspen and sugar maple, initial chemical differences were not correlated with rates of leaf-litter decomposition (King et al. 2001a, 2001b). Furthermore, Finzi and Schlesinger (2002) showed in a free-air CO<sub>2</sub>-enrichment experiment that species composition was more important in explaining litter decomposition rates than growth under elevated CO<sub>2</sub>.

The current study and other in-situ studies (e.g., Rier et al. 2002; Tuchman et al. 2003b) have shown only subtle effects of enriched-CO<sub>2</sub>-induced alterations to leaf-litter chemistry on decomposition and microbial activity. Other studies have demonstrated marked negative effects of altered chemistry on consumption by and growth and development of aquatic invertebrate detritivores utilizing these materials and their attached communities (Tuchman et al. 2002, 2003a, 2003b). The discrepancies among the observed decomposition rates, microbial activities, and detritivore responses might be associated with variable leaching rates of inhibitory compounds from the leaf tissues upon immersion. Relatively short incubations, such as 2 weeks, were clearly adequate to preserve inhibitory compounds in order to suppress biological development of colonizing organisms. With longer incubations in

**Fig. 3.** Microbial activity (mean  $\pm$  1 standard error), including bacterial carbon (*a-c*), bacterial productivity (*d-f*), fungal biomass (*g-i*), and phenol oxidase activity (*j-l*), on leaf litter from three riparian tree species (quaking aspen, sugar maple, and white willow) grown at both ambient (360 parts per million (ppm); solid lines) and elevated (720 ppm; broken lines) concentrations of atmospheric CO<sub>2</sub>.



flowing waters, inhibitory-compound reductions could allow the development of microbial communities and enhanced invertebrate consumption (cf. Golladay et al. 1983). These differences are supported by studies of macroinvertebrate colonization on aspen leaf litter, produced under ambient and elevated CO<sub>2</sub>, which was incubated in a stream for 120 days and showed increasingly disparate results with longer incubation periods (Tuchman et al. 2003b). Other species will surely vary in their responses to growth at elevated

CO<sub>2</sub>. For example, the present study showed that differences in BT carbohydrate-bound tannins between ambient- and elevated-CO<sub>2</sub>-produced willow leaf litter were retained for at least 40 days in the stream. Negative effects on colonizing microbial and consuming detritivores would likely extend for considerable periods of time.

In conclusion, the impact of altered leaf chemistry on riparian trees grown under elevated CO<sub>2</sub> is clearly variable, as this leaf litter is immersed and leached in rapidly flowing

streams. However, other mechanisms could indirectly impact forest streams. For example, this study did not address the fraction of the dissolved organic matter (DOM) pool that is leached from the litter either directly in the stream or through soil–water pathways. DOM constitutes an important fraction of allochthonous carbon entering most stream ecosystems (Wetzel 2001). Since phenolic defense compounds are likely produced in excess by a number of riparian tree species under elevated CO<sub>2</sub> and appear to be readily leached, there is a potential for marked shifts in the recalcitrance of the DOM pool as atmospheric CO<sub>2</sub> increases. This study also did not address potential increases in leaf-litter inputs to forested streams. Trees often produce more leaf litter under elevated CO<sub>2</sub> (e.g., Finzi and Schlesinger 2002), which could influence stream carbon fluxes and stoichiometry. Our results imply that such effects will depend on the species composition of the vegetation and on the kinetics of leaf-litter leaching and decomposition.

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