Contrasting rRNA gene abundance patterns for aquatic fungi and bacteria in response to leaf-litter chemistry

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Contrasting rRNA gene abundance patterns for aquatic fungi and bacteria in response to leaf-litter chemistry

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Abstract. Few investigators have examined simultaneous bacterial and fungal responses to leaf-litter chemistry in fresh water. We tested the hypothesis that bacteria would be more abundant on labile litter with lower concentrations of defensive compounds, whereas fungi would be more abundant on recalcitrant litter. We used quantitative-polymerase chain reaction (qPCR) to measure the abundance of bacterial 16S and fungal 18S ribosomal ribonucleic acid (rRNA) genes and found that these groups responded differently to leaf chemistry. Bacterial 16S rRNA genes were 4\times more abundant on labile than on recalcitrant litter. In contrast, fungal 18S rRNA genes were 8\times more abundant on recalcitrant than on labile litter. Peak bacterial gene abundances on day 6 were related to leaf-litter % bound condensed tannin ($r^2 = 0.38$), and peak fungal gene abundances on day 14 were related to % soluble condensed tannin ($r^2 = 0.49$), % bound condensed tannin ($r^2 = 0.34$), and % lignin ($r^2 = 0.33$). Leaf-litter C:N ratios were not associated with microbial gene abundance. The ratio of fungal 18S:bacterial 16S genes also increased along the 1\textsuperscript{st} axis in a principal components analysis of phytochemical variables. The early peak in bacterial rRNA gene abundance may indicate the role of bacteria in the early decomposition of leaf litter. rRNA gene abundance patterns demonstrate that bacteria and fungi have different patterns of growth and productivity in response to leaf-litter chemistry.

Key words: stream ecology, leaf litter, qPCR, decomposition, 16S rRNA, 18S rRNA, \textit{Populus}.

Bacteria and fungi play important roles in the decomposition of leaves in streams (Petersen and Cummins 1974, Suberkropp and Klug 1974, 1976, Webster and Benfield 1986). This process is central to nutrient cycling and the transfer of energy up trophic chains, particularly in headwater streams that have relatively low rates of photosynthesis and depend on terrestrial leaf litter (Cummins 1974, Vannote et al. 1980). Phytochemical differences (e.g., tannin, lignin) between species of leaves can influence freshwater microbes (Gessner and Chauvet 1994, Baldy et al. 1995, Hieber and Gessner 2002, Marks et al. 2009). Fungal biomass differs among leaf types (Gessner and Chauvet 1994, Baldy et al. 1995, LeRoy et al. 2007, but see Hieber and Gessner 2002, Das et al. 2007) and often is correlated positively with decomposition rate and negatively with tannin and lignin concentrations (Gessner and Chauvet 1994, Maharning and Bärlocher 1996). The relationship between bacteria and leaf type is not as clear. Some studies show higher bacterial biomass on faster decomposing leaves (Das et al. 2007) and leaves lower in lignin (Tuchman et al. 2002), whereas others show little to no influence of leaf type even though rates of decomposition differ significantly (Baldy et al. 1995, Hieber and Gessner 2002). Most
microbial biomass on leaf litter is fungal (Findlay et al. 2002), but the considerably shorter turnover times of bacteria suggest that they may play key roles (Gessner 1997, Hieber and Gessner 2002). Thus, measurements of microbial abundance alone may underestimate the contributions and responses of bacteria and fungi (Gessner 1997, Hieber and Gessner 2002), and measurements of growth rates and productivity (e.g., Elser et al. 2000, Stevenson and Schmidt 2004) may provide a better indication of microbial activity.

Advances in molecular biology provide tools for measuring bacterial and fungal productivity and growth during decomposition (Manerkar et al. 2008, Bärlocher 2010). Quantitative real-time polymerase chain reaction (qPCR) is a promising technique for measuring ribosomal RNA gene abundance and is ideal for determining fungal/bacterial RNA ratios (Fierer et al. 2005, 2009, Strickland and Rousk 2010). Changes in the ratio of fungal/bacterial RNA genes also can indicate when the microbial community switches from dominance by bacteria to dominance by fungi. For example, in a large-scale qPCR-based study, Fierer et al. (2009) found an increasing fungal/bacterial RNA gene ratio along a gradient of soil C:N ratios, a result suggesting that fungal dominance increased in soils with high C:N ratios.

We used cottonwoods (Populus fremontii [Fremont cottonwood] and Populus angustifolia [narrowleaf cottonwood]) grown in a common garden to test the response of bacteria and fungi to differences in leaf chemistry. Populus fremontii has lower levels of defensive compounds than P. angustifolia, and the degree of intraspecific phytochemical variation is high, especially among P. angustifolia genotypes (Whitham et al. 2003, LeRoy et al. 2007). Intraspecific and interspecific differences in tannin, lignin, and C:N ratios affect soil microbial communities and ecosystem processes including aquatic (LeRoy et al. 2007) and terrestrial decomposition and N cycling (Schweitzer et al. 2004, 2008a, b). This variation in phytochemistry allows concurrent study of the effects of leaf chemistry at the species and genotype scales.

We hypothesized that bacterial 16S rRNA gene abundance would be positively associated with labile P. fremontii leaf litter, whereas fungal 18S rRNA gene abundance would be higher on recalcitrant P. angustifolia leaf litter. We further hypothesized that bacterial and fungal RNA gene abundances would be correlated with genotype-level soluble and condensed tannin and lignin concentrations and C:N ratios. Specifically, we predicted that microbial communities on leaf tissue with low concentrations of defensive compounds would be dominated by bacteria because their productivity is associated with labile substrates (Pace and Cole 1994). In contrast, we predicted that microbial communities on leaf litter with higher concentrations of recalcitrant compounds would be dominated by fungi because of their enzymatic capacity to breakdown recalcitrant leaf compounds (Bärlocher 1982, Chamier 1985, de Boer et al. 2005).

**Methods**

**Leaf litter and field collections**

We used leaf litter from known genotypes of P. fremontii and P. angustifolia that encompass the range of phytochemical variation documented in this species (Rehill et al. 2006). We collected and air-dried naturally abscised leaves from a common garden near Ogden, Utah, during autumn 2008. The common garden was planted in the spring of 1991 from cuttings of naturally occurring wild trees along the Weber River, Utah. Trees were genotyped with restriction fragment length polymorphism (RFLP) of 35 specific P. fremontii markers (Keim et al. 1989, Martinsen et al. 2001). We selected 4 genotypes of P. fremontii and 6 of P. angustifolia based on variability in tannin concentrations from green tissue (Rehill et al. 2006).

We measured soluble and bound condensed tannin concentration, lignin concentration, and C:N ratios of naturally abscised leaf litter for each genotype. We measured condensed tannins with the acid butanol assay (Porter et al. 1986) with purified P. angustifolia condensed tannins as standards. We estimated leaf-litter lignin concentration gravimetrically on an Ankom 2000 fiber analyzer (Ankom Technology, Macedon, New York) and %C and %N via combustion with a Costech ECS4010 elemental analyzer (Costech Analytical Technologies, Valencia, California).

**Field study**

We placed litter bags in the headwaters of Oak Creek, Arizona, a perennial 1st-order alkaline stream on the southern edge of the Colorado Plateau (lat 35°02’N, long 111°43’W) with an average annual base flow of 368 L/s (LeRoy and Marks 2006). Water-quality variables were measured with a Hydrolab minisonde (Hydrolab–Hach, Loveland, Colorado) (Table 1).

We created litter bags for each genotype by mixing together the litter of 3 replicated clones. We placed 2 g of this mixture in 20 × 20-cm 6.4-mm-mesh Vexar® bags (Trical netting, Aquatic Eco-Systems, Apopka, Florida). In November 2010, we secured litter bags to rebar stakes that were fixed to the stream bed in areas of natural leaf-litter accumulation. We distributed the litter bags over a distance of ~1 river kilometer in a
Sample processing

In the laboratory, we removed the leaves from the bag and placed them on a sterile wax surface. We took ~25 leaf cores (~0.5 g) randomly from all leaves in a bag. The cores were spatially distributed across the leaf surface to obtain a full representation of the microbial community. We placed the cores in 15 L of RLT buffer, carefully from the rebar, immediately placed them on ice, transferred them to a −20°C freezer, and processed them within 24 h.

DNA extraction and real-time PCR

We ran amplification and real-time fluorescence detections on the 7900HT Real Time PCR System (Applied Biosystems, Grand Island, New York) with the following thermocycling conditions: 3 min at 50°C for UNG (Uracil-N-Glycosylase) treatment, 10 min at 95°C for Taq activation, 15 s at 95°C for denaturation, and 1 min at 60°C for annealing and extension × 40 cycles. We obtained C_t values for each 16S and 18S rRNA gene qPCR reaction with an automatic baseline manual and C_t thresholds of 0.05 and 0.10, respectively, in Sequence Detection Systems software (version 2.3; Applied Biosystems).

Statistical analyses

We log(x)-transformed gene copy numbers prior to analysis. We used Student’s t-tests to compare rRNA gene abundance between P. fremontii and P. angustifolia treatments on each harvest date for bacteria and fungi. We used correlations to assess how much of the variation in peak 16S and 18S gene abundance were explained by initial % soluble condensed tannins, % bound condensed tannins, and % lignin, and C:N ratios among Populus genotypes. We used principal component analysis (PCA) to summarize the variation in phytochemistry data as a composite variable. We
used composite scores for each leaf type in an analysis of covariance (ANCOVA) to compare fungal 18S:bacterial 16S gene ratios on each harvest date. We used harvest date as a fixed effect and the phytochemistry composite score as the covariate. We ran all analyses in SPSS for Windows (release 19.0; IBM SPSS, Armonk, New York).

**Results**

**Phytochemistry**

Initial leaf-litter chemistry differed between species (Table 2). *Populus fremontii* had significantly lower concentrations of soluble condensed tannin ($t = 3.685, p < 0.01$), bound condensed tannin ($t = -6.528, p < 0.01$), and lignin ($t = -7.721, p < 0.001$) than *P. angustifolia*. C:N ratios did not differ between species ($t = 1.316, p = 0.22$). Bacteria and fungi responded with dissimilar patterns of rRNA gene abundance on leaf litter between tree species (Fig. 1A, B) and among genotypes that differed in concentrations of defensive compounds (Figs 2A–D, 3A–D).

**Bacterial 16S gene abundance**

Bacterial 16S gene abundance was highest on labile leaf litter with the highest measured abundances on day 6. The 16S gene copy number was 1.6× greater on *P. fremontii* than on *P. angustifolia* litter ($t = 2.482, p < 0.05$; Fig. 1A). After 14 d, the abundance of 16S genes decreased and remained low and similar on both leaf types for the duration of the study.

**Fungal 18S gene abundance**

Fungal 18S gene abundance was highest on *P. angustifolia* leaf litter. This pattern persisted across all 3 harvest dates. After 6 d of incubation, 18S gene abundance was 4.2× higher on *P. angustifolia* than on *P. fremontii* leaf litter ($t = 0.841, p = 0.20$; Fig. 1B). Fungal 18S gene abundance was highest on both leaf types after 14 d, but *P. angustifolia* supported 6.3×

![Fig. 1. Mean (±1 SE) bacterial 16S (A) and fungal 18S (B) ribosomal ribonucleic acid (rRNA) gene abundance (measured as gene copy number) between *Populus* leaf-litter types during 4 wk of decomposition in a stream. Asterisks indicate significant differences between litter types on a harvest date ($p < 0.05$).](image)
greater fungal gene abundance than *P. fremontii* leaf litter (*t* = −8.16, *p* < 0.001; Fig. 1B). Differences among treatments persisted after 28 d (*t* = −4.55, *p* < 0.001; Fig. 1B), but fungal gene abundance decreased relative to its peak measurement on day 14.

Fungal 18S gene abundance patterns were opposite those of bacterial 16S gene abundance relative to leaf-litter phytochemistry. 18S gene abundance was positively associated with soluble (*r*² = 0.49, *p* < 0.05; Fig. 3A) and bound condensed tannin concentrations (*r*² = 0.34, *p* < 0.05; Fig. 3B). Fungal 18S gene abundances were positively correlated with lignin concentrations (*r*² = 0.33, *p* < 0.05; Fig. 3C), but we found no pattern or significant relationship with leaf litter C:N ratios (*r*² = 0.040, *p* = 0.43; Fig. 3D).

**PCA**

The 1st two axes of the PCA explained 97% of the variation in phytochemistry. Percent soluble condensed tannin, % bound condensed tannin, and % lignin were significantly correlated (*|r|* > 0.85) with the 1st axis (Table 3). Fungal:bacterial rRNA gene ratios increased linearly across the 1st axis. This pattern was consistent among harvest dates (*p* < 0.01). Fungal:bacterial rRNA gene ratios differed significantly among harvest dates (ANCOVA, *F*₂,₂⁹ = 26.04, *p* < 0.01; Fig. 4).

**Discussion**

Our research showed that leaf-litter chemistry mediates the relative abundance of bacteria and fungi throughout decomposition. The early peak in bacterial 16S rRNA gene abundance and lower fungal:bacterial gene ratios suggest that bacteria are more productive on labile leaf litter that has lower concentrations of defensive compounds, especially during early decomposition. On the other hand, patterns of fungal 18S rRNA gene abundance suggest that fungi become increasingly productive on leaf litter with higher concentrations of defensive compounds. It is likely that after the initial mass loss from leaching of soluble C compounds (e.g., Webster et al. 1999) and assimilation of labile leaf tissue by bacteria
during early decomposition, what remains are recalcitrant forms of C available for fungal colonization (de Boer et al. 2005, Romani et al. 2006, Poll et al. 2008). These patterns are attributable to the enzyme capabilities of fungi for degrading recalcitrant compounds (de Boer et al. 2005, Joergensen and Wichern 2008). Consequently, primarily recalcitrant compounds, such as condensed tannin and lignin, may provide the C source for aquatic fungal communities and consumers, such as macroinvertebrates (Graça et al. 1993, Rong et al. 1995).

Patterns of bacterial and fungal gene abundance and the timing of peak gene abundance differ from patterns reported for biomass (Gessner and Chauvet 1994, Baldy et al. 1995, Gessner 1997, Hieber and Gessner 2002). For example, bacterial and fungal biomass peak later in decomposition (e.g., 4–12 wk) (Gessner and Chauvet 1994, Baldy et al. 1995 [Populus-based study], Hieber and Gessner 2002, Das et al. 2007). Biomass may peak later than rRNA, but the qPCR patterns suggest that bacteria contribute to early decomposition when their absolute and relative 16S gene abundances are greatest, reflecting a period of high bacterial productivity.

The positive correlations we observed between fungal gene abundance and condensed tannins and lignin concentrations also are in contrast to other published results (e.g., Gessner and Chauvet 1994, Gessner 1997). The patterns we observed may be the

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**TABLE 3.** Results of a principal components analysis based on the initial phytochemistry of the 4 *Populus fremontii* and 6 *Populus angustifolia* genotypes. Bold indicates statistical significance (*p* < 0.01).

<table>
<thead>
<tr>
<th>Result</th>
<th>Principal component</th>
</tr>
</thead>
<tbody>
<tr>
<td>% variation explained</td>
<td>70.5 26.3 3.0 0.2</td>
</tr>
<tr>
<td>Cumulative % total variance explained</td>
<td>70.5 96.8 99.8 100</td>
</tr>
</tbody>
</table>

**Correlations of original variables with PC1**

| % soluble condensed tannin | 0.86 |
| % bound condensed tannin | 0.99 |
| % lignin | 0.97 |
| C:N | −0.39 |

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**Fig. 3.** Correlations of fungal 18S ribosomal ribonucleic acid (rRNA) gene abundance (measured as gene copy number) and leaf-litter % soluble condensed tannin (A), % bound condensed tannin (B), % lignin (C), and C:N ratios (D). Each point represents the mean 18S rRNA gene copy number on an individual *Populus* genotype on harvest day 6.
result of the specificity and sensitivity of qPCR. These assays can quantify total bacteria and fungi even when target gene numbers are extremely low (Smith and Osborn 2009) and can detect nucleic acid regardless of developmental stage or taxon.

Increases in gene abundance can indicate increases in cell number (Manerkar et al. 2008) or increased cellular activity (e.g., growth, transcription) (Stevenson and Schmidt 2004). We were unable to differentiate these 2 responses, but both are indicative of conditions favorable for growth in bacteria (Elser et al. 2000, 2003, Stevenson and Schmidt 2004) and fungi (Parrent and Vilgalys 2009). The difference between the responses we observed and those reported by other investigators might reflect the fact that qPCR is a direct index of productivity whereas biomass measurements cannot differentiate between active and inactive cells.

Organisms including bacteria increase rRNA gene abundance and rates of rRNA transcription in response to the nutrient content of substrates to meet the demands of biosynthesis and growth (Elser et al. 2000). Correlative and manipulative studies show that rRNA abundance is related to changes in resource availability and that bacteria with more rRNA gene copies respond more quickly (i.e., higher growth rates) to fluctuations in resources than bacteria with fewer rRNA copies (Condon et al. 1995, Klappenbach et al. 2000, Stevenson and Schmidt 2004). Leaf litter provides a pulse of resources, so changes in rRNA gene abundance may occur in response to the ability of fast-growing bacteria with high rRNA gene abundance to out-compete other strains, particularly on labile leaf litter.

The relationship between 18S rRNA gene abundance and fungal activity is more equivocal because of the high degree of variation in gene abundance among cells (Rajala et al. 2011). Fungi with higher gene abundance may have higher rates of growth under favorable conditions (e.g., Parrent and Vilgalys 2009). Fungi must increase enzyme production to access C and nutrients from larger and more recalcitrant molecules in leaf-litter tissue. Biomass may decrease on high-tannin and -lignin leaf types, but the fungal cells that are present may be very active or may have high rRNA gene abundance (higher 18S rRNA gene abundance).

Substrate C:N ratios can influence microbial patterns of growth (e.g., Fierer et al. 2009) and decomposition (Enríquez et al. 1993). However, this relationship is unclear (Chapin et al. 2002, Strickland and Rousk 2010). Like us, LeRoy et al. (2007) found no significant correlation between C:N ratios and decomposition in a study of aquatic leaf litter at the genetic scale. Collectively, these patterns demonstrate that leaf-litter C:N ratios do not control decomposition in some systems. Instead, C quality of the leaf litter appears to influence the decomposer community directly. Gessner and Chauvet (1994) observed a stronger influence of C than N on fungus-based decomposition. In their study, initial leaf-litter lignin content explained ~90% of the variation in decomposition, and N was a very poor predictor. In aquatic systems, microbes may not be constrained by leaf-litter N because bacteria and fungi can access N from the water column (Gulis and Suberkropp 2003).

Competition between bacteria and fungi also may be driving these patterns of rRNA gene abundance. Lower fungal gene abundance and lower fungal:bacterial rRNA gene ratios on labile litter may be a result of competitive exclusion of fungus by bacteria, especially in high-nutrient environments. Fungal biomass in both terrestrial and aquatic ecosystems is higher in the absence than in the presence of bacteria because of competitive release (Mille-Lindblom and Tranvik 2003, Rousk et al. 2008).

Recalcitrant and slow-decomposing organic matter (e.g., P. angustifolia) has been viewed traditionally as being of low quality (e.g., Hobbie 2000), but our data suggest that the bottom-up influence of recalcitrant organic matter may be important in aquatic systems (Pace and Cole 1994). Slow-decomposing leaf litter may feed directly into aquatic food webs by supporting the secondary production of fungi.
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