

A fungal endophyte slows litter decomposition in streams

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SUMMARY

1. Phyllosphere interactions are known to influence a variety of tree canopy community members, but less frequently have they been shown to affect processes across ecosystem boundaries. Here, we show that a fungal endophyte (*Rhytisma punctatum*) slows leaf litter decomposition of a dominant riparian tree species (*Acer macrophyllum*) in an adjacent stream ecosystem.

2. Patches of leaf tissue infected by *R. punctatum* show significantly slower decomposition compared to both nearby uninfected tissue from the same leaf, and completely uninfected leaves. These reduced rates of decomposition existed despite 50% greater nitrogen in infected tissues and may be driven by slower rates of decomposition for fungal tissues themselves or by endophyte–hyphomycete interactions.

3. Across a temperate forest in the Pacific Northwest, approximately 72% of all *A. macrophyllum* leaves were infected by *R. punctatum*. Since *R. punctatum* infection can influence leaf tissue on entire trees and large quantities of leaf litter at the landscape scale, this infection could potentially result in a mosaic of ‘cold spots’ of litter decomposition and altered nutrient cycling in riparian zones where this infection is prevalent.

Keywords: *Acer macrophyllum*, aquatic–terrestrial interaction, leaf litter decomposition, Pacific Northwest, *Rhytisma punctatum*

Introduction

Understanding the dynamic interplay at the interface of terrestrial–aquatic habitats is an important aspect of ecosystem science. Allochthonous inputs of riparian leaf litter are often crucial for ecosystem function and community dynamics in headwater streams (Petersen & Cummins, 1974; Cummins *et al.*, 1980; Vannote *et al.*, 1980; Graça, 2001). Numerous ecologically complex factors in riparian forests may influence stream function through leaf litter fall and litter quality. It has been found that phytochemistry and innate phenotypic differences among tree species (Webster &

Benfield, 1986; Ostrofsky, 1997; Swan & Palmer, 2004; LeRoy & Marks, 2006; LeRoy *et al.*, 2006; Kominoski *et al.*, 2007; Lecerf *et al.*, 2007) and among genotypes within tree species (LeRoy *et al.*, 2007; Lecerf & Chauvet, 2008) can have dramatic influences on aquatic leaf litter decomposition, aquatic fungal biomass accumulation, and macroinvertebrate community structure. It has also been suggested that a variety of terrestrial agents (e.g. drought, herbivory, pathogens, air pollution) may influence leaf litter chemistry and subsequent decomposition, termed collectively ‘afterlife effects’ (Schowalter, Hargrove & Crossley, 1986; Choudhury, 1988; Findlay & Jones, 1990; Findlay *et al.*, 1996; Grime *et al.*, 1996; Bardgett, Wardle & Yeates, 1998; Belovsky & Slade, 2000), although few such studies have been conducted in riparian or aquatic environments. The results of

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terrestrial studies suggest that factors previously unconsidered could have strong impacts on aquatic processes via changes to leaf litter quality or quantity (Schowalter *et al.*, 1991; Chapman *et al.*, 2003; Schweitzer *et al.*, 2005).

Despite the numerous studies examining phyllosphere influences on litter chemistry and several studies examining their influence on terrestrial decomposition, there have been very few studies investigating the strength of phytochemical 'afterlife effects' across ecosystem boundaries (but see Findlay *et al.*, 1996). Fungal endophytes have been found in virtually all plant species (Saikkonen *et al.*, 1998) and have been shown to be important to terrestrial decomposition. In general, endophyte infections in species of grasses tend to slow terrestrial decomposition rates (Tester, 1992; Omacini *et al.*, 2004; Lemons, Clay & Rudgers, 2005; Rudgers & Clay, 2007; Siegrist *et al.*, 2010).

An alternative effect may be shown when certain endophytes cause localized increases in litter quality. For example, infection of *Acer pseudoplatanus* L. leaves by the endophytic fungus *Rhytisma acerinum* Pers. ex. Fr. maintained pre-senescence foliar nitrogen and total carbon following abscission (Gange, 1996). This fungal infection appears to limit translocation prior to abscission, and such phytochemical alterations are likely to persist after leaf senescence. In the case of *Rhytisma*, the fungal tissues must persist throughout the winter in order for reproduction through ascospores on newly emerging maple leaves in the spring. For this reason, we hypothesized that patches of maple litter infected by *Rhytisma* would decompose slower than patches of uninfected litter. While fungal endophytes represent a major influence on leaf litter quality and phyllosphere communities (Saikkonen *et al.*, 1998; Schulz *et al.*, 2002), the extent to which they may influence in-stream leaf litter decomposition is unknown.

We used a common phyllosphere interaction between a fungal endophyte, maple tar spot [*Rhytisma punctatum* (Pers.) Fr.], and big-leaf maple (*Acer macrophyllum* Pursh) in the Pacific Northwest (U.S.A.) to examine how phyllosphere fungal infection affects leaf litter decomposition rates in a small stream. *Rhytisma punctatum* is an endophytic (and pathogenic; Masee, 1913; Tom Hsiang, personal communication) fungus commonly found in the phyllosphere of forests of the Pacific Northwest (Kanouse, 1947) and other locations across the distribution of *A. macrophyl-*

lum (Woo & Partridge, 1969; Hsiang & Tian, 2007). Other species of *Rhytisma* affect other species of *Acer* across their ranges in Europe and Asia. The infection is characterized by discrete patches of light green leaf tissue infected by tar-like stromata which appear in late July (*ibid.*). On senesced leaves, the overwintering fungal spots still appear green with tar-like splotches, even after the rest of the leaf has changed to a rusty brown colour. We hypothesized that the tar-like stromata within infected patches would slow in-stream decomposition of infected tissue compared to nearby uninfected tissue from the same leaf or completely uninfected leaves from the same tree. Infection was based on the physical presence of patches of tar-like stromata; however, it is possible that hyphae were present in uninfected tissues. Similar reductions in decomposition are shown in several terrestrial studies comparing endophyte-infected versus uninfected tissue (Tester, 1992; Omacini *et al.*, 2004; Lemons *et al.*, 2005; Rudgers & Clay, 2007).

Methods

Site description

We conducted this study in the northwest of Olympia, WA, U.S.A., on the 320 ha campus forest reserve of The Evergreen State College (47°04'18.65"N, 122°58'35.72"W, 59 m elevation), which encompasses the drainages of five small (<2 km) first-order streams. Most temperate forest on the reserve is either riparian or within 0.25 km of riparian habitat. The litter decomposition experiment took place in a short, but fast-flowing stream on the campus forest reserve. The stream varies in width between approximately 1 and 2 m within a 30 m wide basin, and the entire c. 1 km length of the stream is contained within the forest reserve.

Litter inputs

To estimate the frequency of *R. punctatum* infection at our study site, leaf litter, twig and cone fall were measured in 40-cm-diameter litter traps in 11 long-term monitoring plots (each 314.5 m²; <http://academic.evergreen.edu/projects/EEON>) during 2006 and 2007. Leaf litter was collected monthly during the 2-year period in a single litter trap per plot to determine average litter contributions to the forest

floor. Litter inputs included big-leaf maple, *R. punctatum*-infected big-leaf maple, and red alder (*Alnus rubra*) leaf litter, Douglas fir (*Pseudotsuga menziesii*) and western hemlock (*Tsuga heterophylla*) needles and twigs, and the reproductive structures (cones, catkins, etc.) of all species. All litter fall was oven-dried at 70° C for 48 h and weighed separately. Leaf litter infected by *R. punctatum* was separated from non-infected tissues to determine the relative amounts of this litter compared to all other litter types.

Leaf chemistry

We collected *A. macrophyllum* leaves just after autumnal abscission. To standardize for leaf litter quality and to isolate the influence of *R. punctatum* on changes to litter chemistry important for leaf litter decomposition, we collected leaves from a single, large *A. macrophyllum* tree. We collected both *A. macrophyllum* leaves with visible *R. punctatum* stromata (hereafter 'infected') and leaves with no visible stromata (hereafter 'uninfected'). From these leaves, we took three types of 15-mm-diameter subsamples of tissue: (i) punches of *R. punctatum*-infected patches of leaf tissue, (ii) punches from uninfected leaf tissue adjacent to patches infected by *R. punctatum*, and (iii) punches from uninfected tissue from uninfected leaves.

Leaf litter punches for initial chemical analyses were air-dried and ground in a Wiley Mill (Thomas Scientific, Swedesboro, NJ, U.S.A.) to 425 µm. Subsamples (25–50 mg) were analysed for nitrogen (N) and phosphorus (P) content using modified micro-Kjeldahl digestion (Parkinson & Allen, 1975) followed by analysis on a Lachat AE Flow Injection Analyser (Lachat Instruments, Inc., Loveland, CO, U.S.A.), using the salicylate and molybdate-ascorbic acid methods, respectively (Lachat Instruments, 1992). We also estimated carbon (C) by combustion (at 500° C for 3 h) of leaf tissue in a muffle furnace (Barnstead International, Dubuque, Iowa, U.S.A.) and assuming that 50% of the organic matter was C (Vogt, 1991).

Leaf decomposition

Five leaf litter punches were weighed fresh and placed within 1-mm-diameter mesh litterbags. Five replicates of each treatment at three sampling dates resulted in a total of 45 litterbags. The small mesh size

of the litterbags excluded most macroinvertebrates from this study, but allowed colonization by bacteria and fungi and thus microbial decomposition. Leaf litter bags were attached to 1 m lengths of metal rod (2 cm diameter) and anchored onto the stream floor. We collected an additional 20 leaf punches of each treatment for fresh to air-dried, oven-dried and ash-free dry mass (AFDM) conversions. The three treatments (*R. punctatum*-infected, uninfected patches near *R. punctatum* patches, and patches from uninfected leaf tissue) allowed us (i) to determine differences in decomposition rates between uninfected tissue on both infected and uninfected leaves, and thus to rule out the hypothesis that *R. punctatum* only attacks leaves of a certain constitutive quality, and (ii) to examine the direct influence of *R. punctatum* on leaf litter decomposition compared to uninfected tissues of two types.

We recovered litterbags at 9, 23, and 77 days after placement in the stream and transported them to the Field Ecology Lab at The Evergreen State College, where they were stored in a refrigerator at 4° C for up to 12 h. We carefully rinsed leaf punches to remove sediment, placed the punches into labelled envelopes, and dried leaf punches in an oven at 70° C for 72 h prior to weighing. We then combusted the leaf litter from each litterbag in a muffle furnace (Box Furnace, Lindberg/Blue M, Asheville, NC, U.S.A.) at 500° C for 1 h to determine AFDM.

Statistical analysis

For an estimate of *R. punctatum* infection frequency in at our study site, percentages of total leaf litter fall were determined for each plot and for each of the five litter types: conifer needles and twigs, alder leaves, maple infected by *R. punctatum*, uninfected maple, and reproductive structures of all species combined. Average litter fall percentages were calculated across all plots and used in pie charts for visual comparison.

For analysis of leaf decomposition rates, we regressed the natural log of the per cent AFDM remaining against day to determine the rate of exponential decay (*k*) (Jenny, Gessel & Bingham, 1949). All exponential regressions were significant at $P < 0.0001$. Because of problems with a lack of independence between *R. punctatum*-infected and nearby infected patches, we compared decomposition rates (*k*) using a resampling analysis of variance

(ANOVA) approach in Resampling Stats (Resampling Stats, Inc., Arlington, VA, U.S.A.). Per cent mass remaining at each sampling date was also analysed using a resampling ANOVA and Bonferroni's correction for multiple comparisons.

Results

In the conifer-dominated temperate forest at our study site, litter inputs consisted predominantly of conifer litter, but with surprisingly large proportions of total litter mass as broadleaf litter. Over 75% (± 8) of all litter inputs were classified as needles or twigs (dominant species *P. menziesii* and *T. heterophylla*). Another co-dominant species, red alder, made up about 4% (± 2) of total litter inputs. However, over 7% (± 2) of all litter fall consisted of maple leaf litter, and of this 73% (± 10) of all maple litter was infected by *R. punctatum*. Overall, a total of 5% (± 2) of all litter fall was infected by *R. punctatum*, and 42% (± 13) per cent of all deciduous leaf litter was infected by *R. punctatum* (Fig. 1). This is especially important since riparian habitats in the region are dominated almost exclusively by these same deciduous species, while uplands are dominated by conifer species. Almost half of all deciduous litter in these riparian habitats could be *R. punctatum*-infected, making it a potentially important factor in decomposition dynamics in broadleaf-dominated areas.

Leaf chemistry

Leaf litter that was infected by *R. punctatum* significantly differed from uninfected tissues in terms of litter C, N, P and C:N ratios (Table 1). Infected tissues

had higher % C than tissues that were adjacent to infected areas on the same leaf ($F_{2,13} = 5.65$, $P = 0.0250$), but were not significantly different from uninfected leaves. *Rhytisma*-infected tissues showed a more than twofold increase in % N compared to uninfected tissues of either type ($F_{2,5} = 1290.67$, $P < 0.0001$) and a 12% increase in % P compared to uninfected tissues of either type ($F_{2,5} = 14.63$, $P = 0.0283$). Finally, given the significant differences in both % C and % N, C:N ratios were significantly lower for *Rhytisma*-infected tissues by over 140% ($F_{2,12} = 494.35$, $P < 0.0001$).

Aquatic decomposition

We hypothesized that *R. punctatum* infection would slow *A. macrophyllum* leaf decomposition due to the presence of stomata and competition with other microbes. Our results demonstrate that infection by *R. punctatum* resulted in significantly slowed leaf litter decomposition rates in a small nearby stream despite the increased % N and decreased C:N ratios of the leaf litter (Fig. 2). Leaf punches taken from patches of *R. punctatum* decomposed 35% less ($k \pm 1$ SE = 0.0088 ± 0.0007) than leaf punches taken from completely uninfected leaves from the same tree (0.0135 ± 0.0009). Additionally, leaf punches taken from patches of *R. punctatum* decomposed significantly less than leaf punches of nearby uninfected leaf tissue from otherwise infected leaves (0.0126 ± 0.0015). Finally, leaf punches taken from uninfected leaves decomposed at the same rates as leaf punches taken from uninfected tissue found on otherwise infected leaves ($P > 0.05$), suggesting that the reduced decomposition was attributable to the

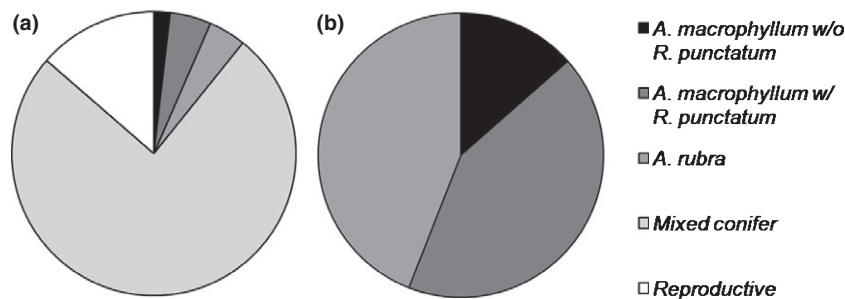


Fig. 1 Average per cent leaf litter fall collected throughout 2006 and 2007 in 11 Evergreen Ecological Observation Network (EON) plots (each 314.5 m²); (a) per cent of total litter fall, including twigs, branches and reproductive structures, and (b) per cent of total deciduous leaf litter fall. Greyscale is ordered in a clockwise direction starting with black. Shades of grey represent the same treatments in each panel.

Table 1 Leaf chemistry for three *Acer macrophyllum* leaf litter treatments: (i) *Rhytisma* patch – leaf punches taken from patches infected by *Rhytisma punctatum*; (ii) nearby patch – leaf punches taken from uninfected leaf tissue directly adjacent to patches of *R. punctatum*; and (iii) uninfected leaf – leaf punches taken from uninfected leaves

Litter treatment	% C	% N	% P	C:N
<i>Rhytisma</i> patch	42.8 ± 0.5 a	2.83 ± 0.02 a	0.61 ± 0.006 a	14.5 ± 0.6 c
Nearby patch	40.1 ± 0.4 b	1.19 ± 0.03 b	0.53 ± 0.011 b	33.6 ± 0.3 b
Uninfected leaf	42.0 ± 0.7 ab	1.12 ± 0.02 b	0.54 ± 0.013 b	37.3 ± 0.6 a

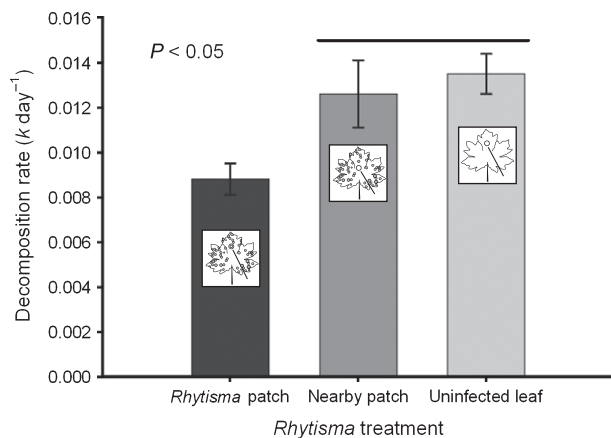


Fig. 2 Decomposition rates ($k \pm 1$ SE) for each of three *Acer macrophyllum* leaf litter treatments: (i) *Rhytisma* patch – leaf punches taken from patches infected by *Rhytisma punctatum*; (ii) nearby patch – leaf punches taken from uninfected leaf tissue directly adjacent to patches of *R. punctatum*; and (iii) uninfected leaf – leaf punches taken from uninfected leaves. Solid bar shows treatments that are not significantly different at $\alpha = 0.05$.

fungus itself, and not to the endophyte-induced changes to whole-leaf chemistry or differences in constitutive foliar quality among leaves prior to infection by *R. punctatum*.

Comparison of per cent mass remaining through time revealed similar patterns at every sampling date. The three *A. macrophyllum* leaf litter treatments were significantly different on sampling day 9 ($F_{2,12} = 4.81$, $P = 0.0293$), day 23 ($F_{2,12} = 8.99$, $P = 0.0041$) and day 77 ($F_{2,12} = 12.19$, $P = 0.0013$). Pairwise comparisons reveal that leaf punches taken from *R. punctatum* patches lost significantly less mass than the uninfected leaves at every sampling date (Table 2), but lost less mass than uninfected tissue on otherwise infected leaves only on days 23 and 77. Uninfected tissues (whether from an otherwise infected leaf or from an uninfected leaf) did not differ in per cent mass loss at any sampling date (Table 2).

Table 2 Per cent mass remaining at each of three sampling dates (9, 23 and 77 d) for three *Acer macrophyllum* leaf litter treatments: (i) *Rhytisma* patch – leaf punches taken from patches infected by *Rhytisma punctatum*; (ii) nearby patch – leaf punches taken from uninfected leaf tissue directly adjacent to patches of *R. punctatum*; and (iii) uninfected leaf – leaf punches taken from uninfected leaves

Litter treatment	Per cent mass remaining		
	Day 9	Day 23	Day 77
<i>Rhytisma</i> patch	91.4 ± 2.3 a	85.4 ± 1.9 a	50.3 ± 1.0 a
Nearby patch	72.3 ± 3.3 ab	61.9 ± 2.6 b	34.7 ± 1.9 b
Uninfected leaf	70.3 ± 0.8 b	60.2 ± 1.7 b	31.4 ± 0.7 b

Measurements represent mean per cent mass remaining ± 1 SE. Lower case letters denote significant differences at $\alpha = 0.05$.

Discussion

Our results suggest three key findings concerning the effects of the terrestrial endophyte, *R. punctatum*, on in-stream litter decomposition. First, although this and previous studies have shown that *R. punctatum* sequesters nutrients in leaf tissue for its own use (Stacey, 1993), these higher levels of N and P do not lead to more rapid decomposition. Instead, the presence of mycelial tissue and tar-like stromata in *R. punctatum* patches may have an inhibitory effect on bacterial or fungal decomposers in the stream environment similar to the competition seen among aquatic hyphomycete species (Bärlocher, 1991). These results corroborate the findings of terrestrial studies showing a deceleration of endophyte-infected leaf decomposition (Tester, 1992; Omacini *et al.*, 2004; Lemons *et al.*, 2005; Rudgers & Clay, 2007). Our results only show the effect of the infection on in-stream decomposition, but further studies could directly examine the *Rhytisma*–microbial or *Rhytisma*–shredder interactions behind the reduced decomposition in streams. Second, there are no significant differences in decomposition rate between

leaves that are completely free of infection from *R. punctatum* and nearby uninfected leaf tissue on an otherwise infected leaf, suggesting a lack of effects induced by *R. punctatum* infection on whole-leaf litter quality traits. This assertion is supported by phytochemical data that show few differences between these two uninfected tissue types, although our phytochemical survey was restricted to only a few litter quality parameters. These results cumulatively suggest that an endophytic phyllosphere interaction in the riparian forest canopy can have a significant influence on in-stream rates of leaf litter processing.

Fungal endophytes are a ubiquitous component of terrestrial ecosystems. In fact, all woody plant species examined are infected by endophytes (Saikkonen *et al.*, 1998). In the Pacific Northwest (U.S.A.), *A. macrophyllum* is a dominant species in riparian forests. Maple litter is considered a recalcitrant litter species because of high quantities of phenolics, lignin and cellulose (Webster & Benfield, 1986), but the infection of this litter type by *R. punctatum* apparently increases its effective recalcitrance. For example, in-stream nutrient release slowed by 35% relative to uninfected tissue. If this infection were relatively rare on the landscape, it would most likely have little influence, but over 70% of all maple litter was infected by *R. punctatum* in 2006–7, and over 42% of all deciduous leaf litter was infected. Although we did not use litter buckets to quantify litter fall in 2010, it has been extremely difficult to find uninfected leaf tissue throughout the campus reserve during this litter fall season. These results suggest that a majority of nutrient release through decomposition of maple litter is mediated by a fungal endophyte, which may have landscape-scale consequences for ecosystem function, both terrestrial and aquatic. Although we have only shown results for aquatic leaf litter decomposition rates, terrestrial and aquatic decomposition rates are largely driven by similar litter characteristics, making the effect of this endophyte potentially wide-reaching.

The study of aquatic–terrestrial interactions encompasses a wide variety of organisms and ecosystem processes and could be expanded through the examination of the landscape-level effects of hidden ecological players like endophytes. Terrestrial studies have examined the influence of interactions between bacterial or fungal endophytes and leaves on terrestrial decomposition processes. In general, the influence of endophytes on terrestrial decomposition is

negative. Tester (1992) found reduced decomposition for endophyte-inoculated corn leaf residue compared to non-inoculated residue in soil incubation studies. Additionally, fescue (*Lolium* sp.) litter exhibited lower litter nitrogen content and decomposed slower when infected by *Neotyphodium* sp. endophytes (Omacini *et al.*, 2004; Lemons *et al.*, 2005; Rudgers & Clay, 2007; Siegrist *et al.*, 2010). Previous studies have also shown that two or more terrestrial agents may interact to influence rates of terrestrial leaf litter decomposition. Herbivore-altered leaves may become more susceptible to certain suites of phyllosphere microbes which, in turn, may influence decomposition rates. For example, aphid-infestation increased colonization by sooty phyllosphere moulds (Pugh & Buckley, 1971; Cox & Hall, 1978; Choudhury, 1986), stimulated phyllosphere microflora through honeydew inputs (Riphagen *et al.*, 1979; Fokkema *et al.*, 1983) and altered dominance between saprophytic and pathogenic fungi on the leaf surface. Choudhury (1986) showed that aphid herbivory on sycamore trees caused an increase in sooty moulds on the leaf surface, which was the hypothesized mechanism for the observed overall decrease in terrestrial decomposition rates of sycamore leaves. Examining interactions such as these that may have cross-ecosystem effects would contribute greatly to our understanding of the role of aquatic–terrestrial interactions in aquatic nutrient cycling.

To appreciate the full implications of phyllosphere influences on aquatic litter decomposition, it will be important to examine how fungal endophytes might interact with aquatic fungi during the leaf breakdown process. It has been shown previously that the in-stream colonization of leaf surfaces by a variety of aquatic fungi can strongly influence shredder feeding activity and leaf decomposition and that microbial conditioning is a crucial step in the decomposition process (Bärlocher, 1980; Bärlocher & Corkum, 2003). It is possible that the presence of *R. punctatum* has an inhibitory effect on the colonization of leaf surfaces by other aquatic fungal and bacterial detritivores. This study did not examine these interactions, but suggests that further investigation into endophyte–hyphomycete, endophyte–bacteria and endophyte–shredder interactions could prove worthwhile.

Our results suggest that terrestrial fungal endophytes can cross ecosystem boundaries to influence in-stream processes. The prevalence of *R. punctatum*

infection in riparian forests of the Pacific Northwest and similar infections in woody plants elsewhere has the potential to influence overall allochthonous carbon inputs to headwater streams and riparian forests and potentially create 'cold spots' of leaf litter processing at the landscape scale (McClain *et al.*, 2003; Kobayashi & Kagaya, 2005). It is possible that this interaction exists in addition to the similarly negative interactions endophytes have with herbivores, whereby plants with endophytes show significantly lower rates of herbivory (Clay, 1990; Saikkonen *et al.*, 1998). Both endophyte-based interactions would then be slowing the carbon cycle and negatively influencing the rate at which nutrients are cycled in ecosystems.

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