3-1-2015

Hemlock Woolly Adelgid Alters Fine Root Bacterial Abundance and Mycorrhizal Associations in Eastern Hemlock

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Citation/Publisher Attribution

JF Vendettuoli, DA Orwig, J Adams Krumins, MD Waterhouse, and EL Preisser. (2015). "Hemlock woolly adelgid alters fine root bacterial abundance and mycorrhizal associations in eastern hemlock." Forest Ecology and Management. 339: 112-116. Available at: http://www.sciencedirect.com/science/article/pii/S0378112714007178.

Running head: Underground effects of adelgid

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KEYWORDS

Adelges tsugae, Tsuga canadensis, mycorrhizae, fine root bacteria, fine root carbon:nitrogen

ABSTRACT

While the impact of aboveground herbivores on plant biomass and fitness has received considerable attention, there has been far less research on the corresponding belowground impacts. The belowground effects of aboveground feeding may be particularly noticeable for invasive and/or outbreaking herbivore species that reach high densities and can cause major damage and sometimes death. The hemlock woolly adelgid, *Adelges tsugae*, is an invasive pest on the eastern seaboard of the United States that feeds on a native shade-tolerant conifer, the eastern hemlock *Tsuga canadensis*. Trees rapidly decline and die following infestation, and the invasion of this insect has devastated hemlock populations from Georgia in the south to Maine in the north. Despite their substantial impact on tree health, we are unaware of any research into the adelgid's effect on hemlock roots and the surrounding rhizosphere. We report the results of research assessing ectomycorrhizal root colonization, rhizosphere bacterial abundance, and root C:N ratios of infested and uninfested *T. canadensis*. We found that adelgid infestation decreased the percentage of root material colonized by ectomycorrhizal fungi by more than 67%. Rhizosphere bacterial abundance on fine roots was 25% lower on adelgid-infested versus uninfested trees, and roots of adelgid-infested trees contained significantly less carbon. Our results demonstrate that aboveground adelgid infestation can affect hemlock root composition and alter belowground interactions with ectomycorrhizal fungi and bacteria. This information demonstrates that above-belowground linkages can transmit the impact of herbivory far from the site of localized damage.

1.0 INTRODUCTION

The impact of insect herbivores on plant growth and community structure can range from inconsequential to major; in extreme cases the structure and functions of entire ecosystems can

47 be substantially altered (Lovett *et al.*, 2006). In some instances, insect herbivores can increase
48 biodiversity via preferential feeding on dominant species, allowing resources to be exploited by a
49 greater number of species (Carson and Root, 2000). In other cases where herbivores inflict
50 substantial damage, outbreaks of such species can devastate their hosts and cause major changes
51 to the environment (Smith and Schowalter, 2001; Gandhi and Herms, 2010) and economic loss
52 (Aukema *et al.*, 2011; Oliveira *et al.*, 2013).

53 Researchers are increasingly aware that herbivore grazing on aboveground green biomass
54 can have profound belowground impacts on the composition of organisms and subsequent
55 nutrient cycling in the rhizosphere (Bardgett and Wardle, 2010). The herbivore removal of
56 aboveground plant tissue can alter patterns of carbon and nutrient allocation in belowground
57 roots (Rasmann *et al.*, 2009). This can affect the composition and abundance of rhizosphere-
58 dwelling organisms. Cattle grazing, for instance, has been shown to alter microbial community
59 composition and food web structure in the root zone of grass (Hamilton and Frank, 2001; Veen
60 *et al.*, 2010). Moose and snowshoe hare grazing have also led to reduced ectomycorrhizal (EM)
61 colonization in roots of balsam poplar (*Populus balsamifera*) and willow (*Salix* spp.) (Rossow *et*
62 *al.*, 1997). These changes can in turn alter rates of nutrient cycling and availability in ways that
63 affect the grazed plant. This feedback loop has been well-documented and can be surprisingly
64 favorable for plant growth and recovery (Ruess and McNaughton, 1987; Krumins, 2014).

65 While the effect of folivory on aboveground-belowground interactions has been well-
66 studied in herbaceous plants, the impact of sap-feeding herbivores on aboveground-belowground
67 interactions in woody plant species has attracted less attention. This gap is notable in light of
68 work documenting that sap-feeder impacts on woody plant fitness equal or exceed those of
69 folivores (Zvereva *et al.*, 2010). Aphid infestations, for instance, indirectly reduce root growth in

Sitka spruce by limiting the tree's ability to provide carbon from photosynthesis (Day and Cameron, 1997) and reduce root tissue density of Douglas fir by inducing the translocation of additional carbohydrates from roots to shoots (Smith and Schowalter, 2001). Herbivory by the needle-feeding scale *Matsucoccus acalyptus* reduced EM colonization of pinyon pine (Gehring and Whitham, 1991); similarly, western spruce budworm (*Choristoneura occidentalis*) defoliation reduced EM colonization in Douglas fir seedlings (Kolb *et al.*, 1999). More generally, both folivores and sap feeders can also impact epiphytic microbial communities through their production of nutrient-rich excrement (Stadler *et al.*, 2001).

The hemlock woolly adelgid (*Adelges tsugae*; 'adelgid') is an invasive sessile herbivore that feeds exclusively on eastern hemlock (*Tsuga canadensis* (L.) Carr.) in the northeastern United States. While it has minimal impact on hemlock health in its native range of Japan and China (Havill *et al.*, 2006), it can kill even mature eastern hemlocks in as little as four years; few heavily-infested trees survive longer than ten years (Orwig and Foster, 1998). While the impact of the adelgid on hemlock physiology has been studied (Radville *et al.*, 2011; Gómez *et al.*, 2012; Gonda-King *et al.*, 2012; Domec *et al.*, 2013), its effects on hemlock root physiology and the associated rhizosphere remain unexplored.

We report the results of work exploring the belowground impact of adelgid infestation on *T. canadensis*. We measured root EM associations of infested and uninfested hemlock trees, rhizosphere bacterial abundance, and root C:N ratios. Our findings demonstrate that aboveground adelgid infestation of eastern hemlock has belowground consequences that likely augment the ecosystem-level impact of this pest and may need to be addressed for the maximal success of forest restoration efforts.

2.0 METHODS

As part of a long-term research program addressing the impacts of the hemlock woolly adelgid on eastern hemlock, we characterized adelgid infestation level and stand vigor in 79 stands in CT and 63 stands in MA. Following their initial characterization (1997-1998 in CT, 2002-2004 in MA; Orwig *et al.*, 2002; Orwig *et al.*, 2012), these stands were repeatedly surveyed for adelgid infestation and stand vigor in 2005, 2007, 2009, and 2011 (Preisser *et al.*, 2008; Preisser *et al.*, 2011). We assessed the rhizosphere of eastern hemlocks in a subset of the stands described above. In order to explore how adelgid infestation affects rhizosphere processes in eastern hemlock, we characterized both fine-root EM colonization (Study #1) and bacterial abundance (Study #2).

In Study #1, we quantified EM colonization of hemlock roots in three adelgid-infested and two uninfested hemlock stands in 2003; we focused our research on ectomycorrhizae as opposed to arbuscular (endo) mycorrhizae because conifers like hemlock are almost exclusively colonized by ectomycorrhizae (Smith *et al.*, 1997). Infested stands were located in south-central Connecticut, and had been colonized by adelgid for 3-10 years; uninfested forests were located in central Massachusetts (Fig. 1). In Study #2, we quantified rhizosphere bacterial abundance from ten infested and ten uninfested hemlock stands in central and northern Massachusetts in 2013 (Fig. 1). In both studies, trees sampled in the 'infested' treatment were heavily infested themselves (>1 mature adelgid/cm foliage growth) and surrounded by other heavily-infested trees. In contrast, trees sampled in the 'uninfested' treatments were in stands where no adelgid had been detected during previous large-scale surveys; to ensure that the sampled trees had maintained their uninfected status, we carefully surveyed each sampled tree and all trees within 10m of it to ensure the absence of adelgid. Because of logistical constraints, we were unable to sample the same sites in both surveys. Regardless of location, all sampled stands consisted of

116 >50% hemlock canopy cover within 100m of the sampled trees, and 100% canopy cover within
117 10 m of the sampled trees.
118 *2.1. Study 1: Ectomycorrhizal colonization of hemlock roots*
119 From each of the five sites, we collected roots from 3–8 hemlock saplings (1.5-2m in
120 height); we sampled a total of 14 saplings from infested sites and 16 from uninfested sites. Soil
121 and stand traits were sampled in the course of several related studies (Cobb *et al.*, 2006; Orwig *et*
122 *al.*, 2013); infested sites averaged 708 $\pm$ 19 [SE] trees ha^{-1}, with a mean hemlock stand basal area
123 of 46.6 $\pm$ 2.7 m^2 ha^{-1}; uninfested sites averaged 1072 $\pm$ 97 [SE] trees ha^{-1}, with a mean hemlock
124 stand basal area of 45.6 $\pm$ 2.0 m^2 ha^{-1}. soils in infested sites had a mean organic (forest floor) soil
125 C:N ratio of 26.1 $\pm$ 2.3% [SE] and a mean mineral soil c:n ratio of 23.2 $\pm$ 0.65%. Soils in
126 uninfested sites had a organic (forest floor) soil C:N ratio of 26.9% and a mineral soil c:n ratio of
127 24.1% (also see online appendix #1). Only trees growing under a hemlock-dominated canopy
128 were sampled. Each sapling was uprooted to expose the entire root system, and three 20cm root
129 samples per tree were collected and rinsed with deionized water (four of 30 sampled trees only
130 had sufficient roots for two 20cm root samples). Although we would have liked to sample mature
131 trees, the labor involved in uprooting multiple large hemlock trees (necessary to ensure that the
132 sampled roots in fact belonged to the chosen tree) necessitated using saplings. Each root sample
133 was assigned a random number to ensure an unbiased assessment and then trimmed down to a
134 5cm section. The grid intercept method (Giovanetti and Mosse, 1980) was used to assess the
135 percent EM colonization for each root sample. Roots with EM colonization were differentiated
136 on the basis of morphology, color, characteristics of the surface of the hyphal mantle, and planar
137 views of different mantle layers using standard methods (Agerer, 1992). Each root sample was
138 randomly dispersed in a 9cm diameter petri plate with 0.5cm grid lines. The intersection between

grid lines and roots were designated as either EM-colonized or non-mycorrhizal. The proportion of root counts that were mycorrhizal was calculated for each root sample and averaged for each sapling. We took a total of 5,902 counts, an average of 197 counts per tree (each tree had a minimum of 100 counts).

2.2 Study 2: Bacterial abundance in the rhizosphere

We collected fine roots (≤ 2mm diameter; Robertson *et al.*, 1999) from three understory hemlocks (2 – 5m tall) at each of twenty sites (10 infested and 10 uninfested); all sampled trees were growing under a hemlock-dominated canopy. Again, our choice of the sampled trees was motivated by the difficulties inherent in uprooting multiple large hemlock trees (necessary to ensure that the sampled roots in fact belonged to the chosen tree). Infested sites averaged 1312 $\pm$ 240 [SE] trees ha^{-1}, with a mean hemlock basal area of 33.2 $\pm$ 1.5 m^2 ha^{-1}; uninfested sites averaged 859 $\pm$ 78 [SE] trees ha^{-1}, with a mean hemlock basal area of 38.2 $\pm$ 1.7 m^2 ha^{-1}. Soils in infested sites had a mean organic (forest floor) soil C:N ratio of 26.9 $\pm$ 1.4% [SE] and a mean mineral soil c:n ratio of 24.5 $\pm$ 2.0%. Soils in uninfested sites had a mean organic soil C:N ratio of 28.8 $\pm$ 1.6% and a mean mineral soil c:n ratio of 24.5 $\pm$ 1.6% (also see online appendix #1; for a more detailed site description, see Orwig *et al.*, 2012). Roots were collected from each hemlock by lightly scraping away leaf litter and organic soil from the base of the tree, extracting the roots, and clipping three 12-15cm root samples. Root samples were combined in a single plastic bag (one bag per tree), immediately returned to the lab, and refrigerated for <2 hours prior to fixation. Before fixing each sample, loose debris and soil was manually shaken off; ~0.1g of fine roots from each tree was then placed in 5mL of phosphate buffer saline (PBS) solution and vortexed for 2 minutes to suspend the bacteria. Each suspension was fixed with 1.5% filtered

161 formalin (1.5% final formalin concentration) and again vortexed. Fixed samples were stored at
162 4^{o}C prior to staining and enumeration.
163 Staining took place within three days of the sample being fixed and collected; because the
164 samples were fixed immediately after collection, there were no time-related differences between
165 bacterial counts taken on different days. Bacterial abundance was determined using acridine
166 orange direct counts (AODC) (Kepner and Pratt, 1994). Cell concentration was optimized by
167 dilution to achieve countable samples. Between 0.1-0.5ml of each fixed sample was removed
168 (the extracted amount was supplemented with PBS to ensure a total volume of 1ml) and then
169 stained with 200µl of 0.1% acridine orange. Each sample was vacuum-captured onto a 0.2µm
170 black polycarbonate filter (EMD Millipore Corporation, MA) and fixed to a slide with
171 immersion oil. Enumeration was done using a Nikon Eclipse Ti inverted fluorescent microscope
172 fixed with a Nikon DS-Fi1 camera. We determined mean bacterial abundance per slide by
173 photographing ten randomly-chosen locations on each slide, using ImageJ (Schneider et al.
174 2012) to count bacterial densities, and averaging the ten counts. Each count thus reflects the
175 mean number of cells per g fresh weight of plant material. Because the extraction procedures
176 involved washing and removing fresh plant tissue, we could not calculate the dry weight of plant
177 material.
178 We used the procedures outlined above to collect an additional fine root sample in 2013
179 for carbon and nitrogen analysis from each tree used for bacterial abundance at the 20 sites. Fine
180 roots were rinsed with deionized water to remove soil particles and dried at 60°C in an oven for
181 48 hrs. Dried samples were ground into a fine powder using a grinding mill (Spex Mixer Mill
182 8000M, Metuchen, NJ) and analyzed for carbon and nitrogen content with a nutrient analyzer

183 (Elementar vario MICRO cube, Mount Laurel, NJ). Two replicates of each fine root sample were
184 analyzed, and the results averaged to calculate a site-level mean.
185 *2.3 Statistical Analysis:* To test the effect of adelgid infestation on EM colonization,
186 bacterial abundance, and root C:N, all samples were grouped by site and the infested and control
187 sites were compared. Because the data from Study #1 did not meet the assumptions of normality,
188 we analyzed it using a non-parametric median test; data from Study #2 was analyzed using one-
189 way ANOVA. EM colonization was calculated as percent colonization and bacterial abundance
190 was calculated as number of cells per gram fresh weight of root. All analyses were performed
191 using JMP 9.0 (SAS, Cary, NC).
192 **3.0 RESULTS**
193 The percentage of EM colonization was significantly lower (10.6$\pm$2.4% [SE]) on infested
194 hemlock roots compared to roots from uninfested trees (32.4$\pm$10.2%) (Median Test; p=0.046).
195 Hemlock fine root bacterial abundance, measured in millions of cells per gram, was also lower
196 on infested versus uninfested trees ($F_{1,18}$=2.22, p=0.044; Fig. 2A). Chemical analysis of fine
197 roots from adelgid-infested versus uninfested trees revealed that percent carbon was significantly
198 lower in infested hemlock stands ($F_{1,18}$=5.11, p=0.036; Fig. 2B), but that adelgid infestation did
199 not affect percent nitrogen (Fig. 2C). Despite the differences in root C, roots from infested versus
200 uninfested stands did not differ in their root C:N ratio (Fig. 2D).
201 **4.0 DISCUSSION**
202 We found that aboveground infestation by hemlock woolly adelgid significantly affected
203 rhizosphere processes. The rhizosphere surrounding fine roots of adelgid-infested trees had less
204 ectomycorrhizal colonization and lower bacterial abundance (Fig. 2A), while the fine roots
205 themselves had lower carbon concentrations (Fig. 2B). The lower percentage of ectomycorrhizal

206 colonization found in our results is consistent with findings following herbivory from several
207 types of insects (Gehring and Whitham, 1994b). One of the most common causes of decreased
208 ectomycorrhizal colonization is herbivory-driven reductions in photosynthate availability; this
209 can disrupt carbohydrate supply to mycorrhizae and reduce mycorrhizal root tip abundance
210 (Gehring and Whitham, 1994b) and mycorrhizal inoculum potential (Lewis *et al.*, 2008). Aphid
211 outbreaks on other conifer species, for instance, can decrease photosynthetic efficency (Day and
212 Cameron, 1997). The hemlock woolly adelgid has been shown to cause similar reductions in
213 photosynthesis (Nelson *et al.*, 2014), and continued feeding on hemlock likely leads to
214 continuous carbohydrate depletion. It is possible that the reduction in carbon found in the fine
215 roots of infested trees (Fig. 2B) and lower EM colonization result from a disruption of that
216 carbon source.
217 Environmental conditions can also play a significant role in the mycorrhizal response to
218 herbivory (Bardgett and Wardle, 2003; Gehring and Whitham, 2003). There has been a
219 documented decrease in EM colonization in sites with higher soil nutrient status compared with
220 water- and nutrient-stressed sites (Gehring and Whitham, 1994a). Several environmental
221 characteristics of chronic HWA infestation may have impacted the EM colonization response
222 seen in this study. First, Stadler et al. (2005; 2006) provided evidence that adelgid impacts the
223 composition of throughfall in infested stands. Their work showed higher inputs of N into the soil
224 under adelgid-infested trees. Second, in addition to inputs from throughfall, adelgid damage
225 often yields microenvironmental conditions that lead to increased soil N due to changes in
226 decomposition, N cycling and availability, and reduced tree uptake of nutrients (Kizlinski et al.
227 2002; Orwig et al. 2008). Enhanced soil N status resulting from either of these mechanisms may
228 also have led to reduced colonization of hemlock fine roots and associated bacterial levels.

Finally, altered mycorrhizal community structure resulting from increased N in the system may have selected for ectomycorrhizal species that perform less of a service for their host but still require the same carbon cost (Johnson, 1993).

We expected the abundance of bacterial cells colonizing the roots of adelgid-infested trees to be higher. Experiments in grasslands ecosystems showed that grazed plants exude more carbon into the rhizosphere, thus stimulating microbial growth and metabolism (Hamilton and Frank, 2001). The priming effect (Kuzyakov *et al.*, 2000) stimulated by above-ground herbivory fed back positively to the grasses, increasing nutrient availability. Rhizosphere microbes have the primary responsibility for making nutrients available to plants through the decomposition and mineralization of soil organic matter (Vessey, 2003). It is interesting to note that we did not find increased microbial abundance in the trees we studied. This difference may be due to important differences in the feeding behavior of herbivores and how they affect the host plant (Lovett *et al.*, 2006). Sucking insects, for instance, export far less wasted plant biomass (inreasing soil organic matter) and frass to the forest floor than do chewing insects or larger grazers (Zvereva *et al.*, 2010).

Bacterial and fungal communities are often tightly coupled in the rhizosphere. The reduced bacterial abundance found in adelgid-infested hemlock stands may simultaneously be linked to reduced ectomycorrhizal associations and changes in root and soil nutrient chemistry associated with infestation. Indeed, some studies have found that soil communities experiencing mycorrhizal loss also lose their fungally-associated bacteria (Hol *et al.*, 2014). However, the significant decline in absolute numbers of bacteria suggests resource limitation from the root. The implications of decreased bacterial load will be decreased mineral nutrient availability to the tree (Vessey, 2003; Wardle *et al.*, 2004); this is consistent with our results (Fig. 2A). Knowing

both the load of EM and bacteria in the rhizosphere is, however, only the first critical step. Soil communities are classically divided into bacterial- and fungal-based energy channels (Moore and Hunt, 1988; De Ruiter *et al.*, 1995). It is the balance of these two energetic pathways, however, that leads to stability and functioning of the rhizosphere community (Rooney *et al.*, 2006) and overall plant health. Therefore, future work will test differences in community composition of both mycorrhiza and bacteria colonizing the roots of trees in affected and unaffected sites. The reduced colonization by EM and bacterial abundance found here may be caused by strong competitors dominating the rhizosphere community.

Our work provides the first documentation of the below-ground consequences of above-ground herbivory on eastern hemlock by an exotic herbivore. The impact of aboveground feeding by the hemlock woolly adelgid on the rhizosphere processes of the hemlock and the mycorrhizal and bacterial abundances illustrates the need for a greater understanding of how herbivores impact all aspects of an ecosystem. From a management perspective, there is increased recognition (Kardol and Wardle, 2010) of the importance of aboveground-belowground linkages in determining the efficacy of management and restoration efforts. Specifically, adelgid-mediated alterations in the belowground communities that facilitate hemlock growth could make it more difficult to replant hemlocks in formerly-suitable areas. Further research should investigate the soil community structure of infested hemlock stands against uninfested hemlock stands, to see if there are shifts in the species found in addition to the change in abundance of bacteria.

5.0 ACKNOWLEDGEMENTS

We thank M. Patel for her assistance with the lab work and C. Renaud for help processing samples. The manuscript was greatly improved by by the comments and suggestions

of two anonymous reviewers. This work was funded by RI00HI-4004 to EP, NIFA 2011-67013-30142 to EP and DO, NSF funding through its programs in Research Experience for Undergraduates (DBI 10-03938), Long Term Ecological Research (DEB 12-37491), and is a publication of the Harvard Forest Long Term Ecological Research (LTER) Site.

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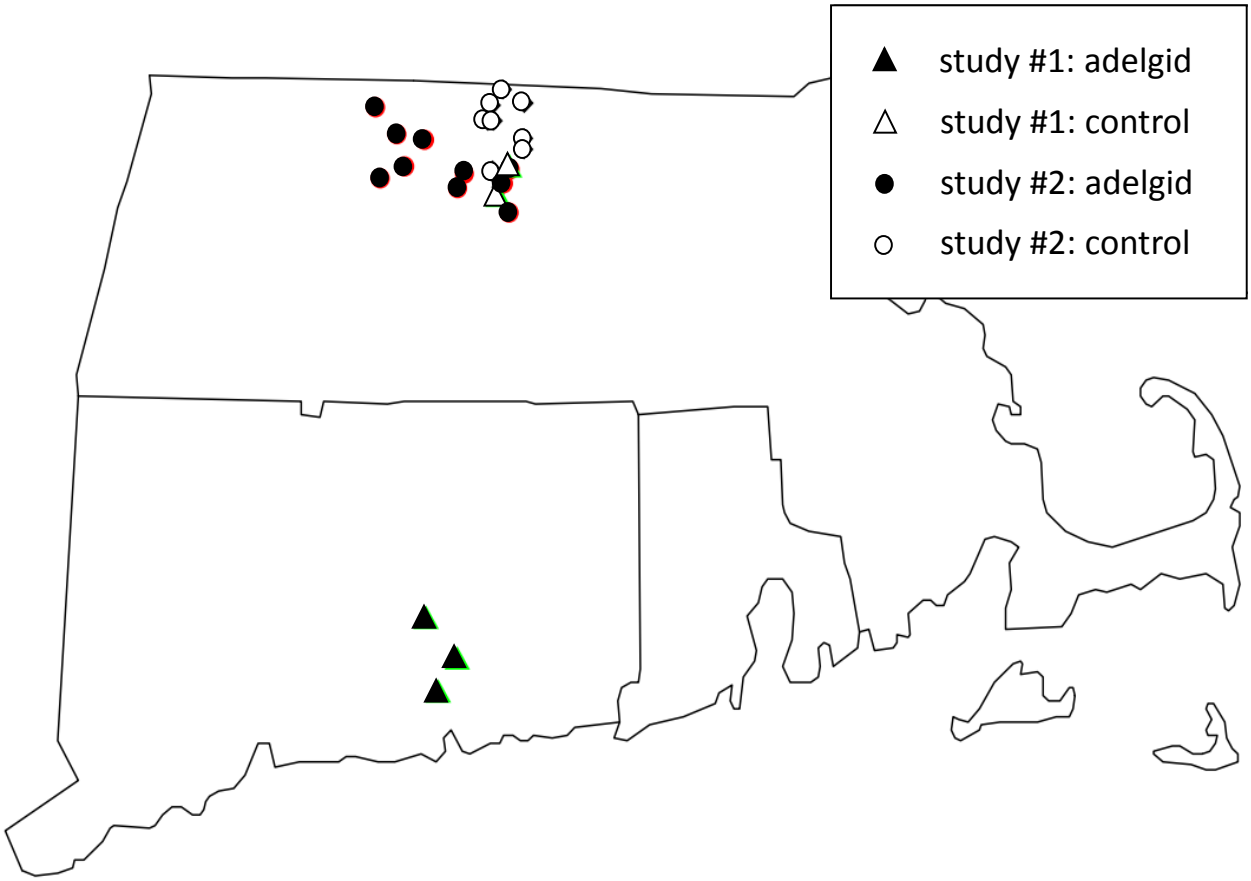
403

7.0 Figure Legends

Figure 1. Sites in Connecticut and Massachusetts used for the mycorrhizal (Study #1) and root bacterial abundance (Study #2) surveys.

Figure 2. Mean bacterial abundance (A), mean percent carbon (B), mean percent nitrogen (C), and mean carbon:nitrogen ratio (D) of eastern hemlock fine roots in 10 uninfested hemlock stands and 10 adelgid-infested hemlock stands.

411 **7.1 Figure 1.**



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414 **7.2 Figure 2.**

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