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Research article

Spatial and Temporal Distribution of Imidacloprid Within the Crown of Eastern Hemlock

Richard M. Turcotte,[1] Anthony Lagalante,[2] Jonathan Jones,[2] Frank Cook,[2] Thomas Elliott,[3,*] Anthony A. Billings,[4] and Yong-Lak Park[5,6]

[1]USDA Forest Service, Forest Health Protection, 180 Canfield Street, Morgantown, WV 26505, [2]Department of Chemistry, Villanova University, Villanova, PA 19085, [3]Plant Protection and Quarantine USDA Animal Plant Health Inspection Service, Morgantown, WV 26505, [4]Department of Statistics, West Virginia University, Morgantown, WV 26506, [5]Division of Plant and Soil Science, West Virginia University, Morgantown, WV 26506, and [6]Corresponding author, e-mail: yopark@mail.wvu.edu

*Deceased

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Abstract

Systemic imidacloprid is the most widely used insecticide to control the hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), an exotic pest of eastern hemlock, *Tsuga canadensis* (L.) Carriére in the United States. This study was conducted to 1) determine the effect of treatment timing (spring vs. fall) and application method (trunk injection vs. soil injection) on the spatial and temporal distribution of imidacloprid within the crown of *A. tsugae*-free eastern hemlock using a competitive enzyme-linked immunosorbent assay (ELISA), 2) compare ELISA to gas chromatography-mass spectrometry (GC/MS) for the detection of imidacloprid in xylem fluid, and 3) determine the concentration of imidacloprid in leaf tissue using high performance liquid chromatography with tandem mass spectrometric (LC/MS/MS) detection methods. Xylem fluid concentrations of imidacloprid were found to be significantly higher for spring applications than for fall applications and for trunk injections than soil injections in the first year posttreatment. A total of 69% of samples analyzed by ELISA gave 1.8 times higher concentrations of imidacloprid than those found by GC/MS, leading to evidence of a matrix effect and overestimation of imidacloprid in xylem fluid by ELISA. A comparison of the presence of imidacloprid with xylem fluid and in leaf tissue on the same branch showed significant differences, suggesting that imidacloprid moved intermittently within the crown of eastern hemlock.

Key words: hemlock woolly adelgid, Imidacloprid, ELISA, insecticide distribution

Eastern hemlock, *Tsuga canadensis* (L.) Carriére (Pinales: Pinaceae), is an important component of both the urban and forest landscape of the eastern United States. It is a long-lived, shade-tolerant species that strongly influences its environment and other species. The dense evergreen canopy along with its ability to grow in nearly pure stands creates a distinct microclimate that is important for a wide variety of plant and animal species.

The hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), is the single greatest threat to the health and sustainability of hemlock as an urban and forest resource in eastern North America (Knauer et al. 2002). This exotic insect is currently established in 18 eastern states in the United States (USDA 2014) where it causes tree decline and mortality. *A. tsugae* reproduces parthenogenetically (an all-female population with asexual reproduction), and has six stages of development (egg, four nymphal instars, and adult) and two generations a year on hemlock (McClure 1989). This adelgid (~1 mm) settles on young twigs at the base of the hemlock needle and feeds on the parenchyma cells of the xylem rays that transfer and store nutrients (Young et al. 1995, McClure et al. 2001). All ages of hemlock, from seedling to mature trees, are fed upon. Feeding from *A. tsugae* can kill a mature tree in about 5–7 years (McClure 2001).

Imidacloprid (1-[6-chloro-3-pyridinyl) methyl]-*N*-nitro-2-imidazolidinimine), a systemic insecticide, is effective against a wide variety of sap-sucking insect pests on a wide variety of crops. It has a mode of action similar to that of the botanical product nicotine, functioning as a fast-acting insect neurotoxicant that binds to the nicotinergic receptor sites in the postsynaptic membrane of the insect's nerves, mimicking the action of acetylcholine. As a result, the heightening, then blocking of the firing of postsynaptic receptors occurs with increasing doses (Schroeder and Flattum 1984). Because imidacloprid is slowly degraded in the insect, it causes substantial disorder within the nervous system, leading in most cases to death (Mullins 1993, Smith and

Krischik 1999). As a result the chemical has become one of the world's most widely used insecticides (Silcox 2002, Jeschke and Nauen 2008). Imidacloprid is sold under a variety of tradenames (e.g., Admire [Bayer, Kansas City, Missouri], Advantage [Bayer, Shawnee Mission, Kansas], Gaucho [Bayer, Monheim, Germany], Premise [Bayer, Leverkusen, Germany], and Touchstone [Bayer, Research Triangle Park, North Carolina]) and has 140 crop uses (Jeschke et al. 2010). It is both a systemic and contact insecticide (Mullins 1993) and has become the preferred pesticide for controlling *A. tsugae* (Smith and Lewis 2005, Eisenback et al. 2009).

Several methods have been developed for quantifying the amount of imidacloprid present in treated plants. Enzyme-Linked Immunosorbent Assay (ELISA) is a common and relatively inexpensive method (Cowles et al. 2006) used to detect imidacloprid in eastern hemlock sap and tissue (Cowles et al. 2006, Eisenback et al. 2009, Dilling et al. 2010). In this assay, imidacloprid residues in a sample compete with enzyme (horseradish peroxidase [HRP])-labeled imidacloprid for a limited number of antibody binding sites on the inside of the test well (EnviroLogix 2004). The levels of bound conjugate are determined spectrophotometrically and, as with all ELISA tests, the sample concentrations are inversely proportional to the color development. A micro-titer plate reader and software are then used to measure end-point absorbance at 450 nm to determine the level of insecticide. Other analytical techniques such as gas chromatography and mass spectrometry (GC/MS) analysis have been found to be selective and sensitive for determining imidacloprid in soil and plant tissue (Rouchaud et al. 1996, McDonald and Meyer 1998, Tattar et al. 1998, Jones 2007). Liquid chromatography (LC) coupled with mass spectrometry in tandem (LC/MS/MS) is another widely used method and is used by the USDA AMS standards lab in Gastonia, NC to detect pesticides in plant tissue (Li and Li 2000, Di Muccio et al. 2006, Cook 2008).

Numerous factors likely impact the movement and distribution of imidacloprid. The effect of application method (Tattar et al. 1998, Cowles et al. 2006), season, and the movement of imidacloprid throughout the wood and needle tissue of the crown of *A. tsugae*-infested eastern hemlock have been examined in other studies (Eisenback et al. 2009, Dilling et al. 2010). Induced plant responses to insect feeding are well documented and can have a significant impact on the physical and biochemical systems of plants (Haukioja 1991, Nykänen and Koricheva 2004, Karban and Baldwin 2007, Radville 2011). *A. tsugae* feeding can quickly lead to needle loss, resulting in dieback (Cheah et al. 2004), restrictions in the uptake and movement of water (McClure 1995), and reduced new growth (McClure 1991). However, none of the previous studies investigated the movement of imidacloprid on *A. tsugae*-free eastern hemlock. Due to the difficulty in detecting *A. tsugae* at low densities (Evans and Gregoire 2007), imidacloprid is sometime used as a preventative treatment measure for high value trees and stands that are at risk from *A. tsugae*. Therefore, investigating the movement of imidacloprid on *A. tsugae*-free hemlock with similar live crown ratios (LCRs) allows one to investigate the temporal and spatial distribution of imidacloprid without the confounding factors of *A. tsugae* being present and different sized crowns.

This study was conducted to: 1) determine the effect of treatment timing (spring vs. fall) and application method (trunk injection vs. soil injection) on the spatial and temporal distribution of imidacloprid within the needles in the crown of uninfested eastern hemlock using a competitive ELISA, 2) compare ELISA to GC/MS for the detection of imidacloprid in xylem fluid, and 3) determine the concentration of imidacloprid in leaf tissue by LC/MS/MS.

Materials and Methods

Study Sites

This study was conducted at two *A. tsugae*-free and previously untreated sites located in Monongalia County, West Virginia in 2005 and 2006. Site A was located at the West Virginia University Forest (39° 39′ 22.80″ N, 79° 45′ 04.33″ W) within a 13-ha stand of eastern hemlock, and Site B at a 16-ha stand located at the West Virginia Botanic Garden (39° 37′ 41.50″ N, 79° 51′ 52.45″ W). The hemlock stand at site A was considered to be an oak-hemlock forest. The stand's overstory included *Acer rubrum L., Betula lenta L., Liriodendron tulipifera L., Nyssa sylvatica Marshall, Quercus alba L., Quercus coccinea* Münchhausen, *Quercus prinus* L., *Quercus rubra* L., and *T. canadensis*. The stand's understory was primarily dominated by *A. rubrum, B. lenta, N. sylvatica*, and *T. canadensis*. Almost half of the understory was comprised of eastern hemlock. The hemlock stand at site B consisted of a variety of species comprising the overstory canopy. The overstory canopy included *A. rubrum, Fagus grandifolia Ehrh., L. tulipifera, N. sylvatica, Oxydendrum arboreum (L.), Q. alba, Q. rubra*, and *T. canadensis*. Nearly two-thirds of the overstory was comprised by oak, yellow-poplar, and Eastern hemlock. The understory consisted of *A. rubrum, B. lenta, F. grandifolia, L. tulipifera, N. sylvatica, O. arboreum, Q. rubra*, and *T. canadensis*. Eastern hemlock and red maple comprised two-thirds of the understory species composition.

A total of 32 single-stem hemlock trees were randomly selected from the hemlock stands with LCR of >80% at each site. The minimum between-tree distance was 9.1 m. For each tree, dbh (diameter at 1.37 m above the ground) and tree height were recorded. Trees were blocked by dbh so similar sized trees were present in each treatment class. The mean values (±SD) of dbh (cm) and height (m) for eastern hemlock at site A were 21.9 ± 6.7 and 13.5 ± 3.9, respectively, and were 24.4 ± 7.2 and 11.8 ± 1.9, respectively, at site B.

To ensure all hemlock trees were *A. tsugae* free, stand-wide surveys of hemlock trees to check the presence of *A. tsugae* at each site were conducted in 2004–2006. In addition, bi-weekly visual surveys of the distal 45 cm of one randomly selected branches from each tree were conducted (ground level to ~3 m above ground) on all study trees from May to October in 2005 and 2006 (Costa 2005, Costa and Onken 2006, Turcotte 2016).

Insecticide Application Methods

At each site, trees were treated with Merit 2F (Bayer, Kansas City, Missouri) imidacloprid soil injection treatment (0.86 g a.i. in 30 ml/2.5-cm dbh, $n = 8$) and trunk injection treatment with an Arborjet Tree I.V. system (IMA-jet 5% in 4 ml/2.5-cm dbh, $n = 8$) at label rates (Doccola et al. 2007) in the spring (2 May) and fall (10 October) of 2005. Soil injection treatments were made using a Kioritz applicator (Kioritz Corp., Tokyo, Japan) using the basal system (Silcox 2002, Dilling et al. 2010) with the footplate set at 12.7 cm (Turcotte et al. 2008a).

Branch Sampling and Sample Processing

To monitor the movement of imidacloprid within a tree, each tree was divided into four cardinal directions and three height sections at 2.4–4.8 m (lower), 5.1–7.3 m (middle), and 7.6–9.7 m (upper) (i.e., a total of 12 branch samples per tree). All samples were from the tip of the branch ~ 61 cm in length and were collected using a telescoping pole pruner (Hasting HV-240, Hasting, Michigan), placed in polyethylene bags packed in ice, transported to the laboratory, and stored in a freezer at 4 °C until the xylem fluid and leaf tissue could be extracted. The sampling was done at 3, 9, 15, 21, and

52 weeks posttreatment. All branch samples collected were examined for the presence of *A. tsugae*. Samples were stored for a minimum of six months and no more than 3 years. Xylem fluid was extracted using a 61-cm pressure chamber (PMS Instrument Co., Albany, Oregon). The cut end of each hemlock branch was trimmed and the cambium layer removed. This end was inserted through a rubber gasket and the entire branch was placed within the pressure chamber. The chamber was gradually pressurized with nitrogen up to 4.14 MPa. Between 500 and 1,000 µl of expressed xylem fluid was collected with a micropipette, placed in a 1.5-ml microcentrifuge tube and refrigerated at 4 °C.

Imidacloprid Concentration in Xylem Fluid

Concentration of imidacloprid within xylem fluid was determined using a competitive ELISA test kits (EP-006, EnviroLogix, Portland, Maine). Its 96-well test plates, imidacloprid residuals in samples compete with HRP-labeled imidacloprid enzyme for a limited number of antibody binding sites on the inner surface of the well (Lagalante and Greenbacker 2007). The plates were washed, and the outcome of the reaction was visualized by a color development stage (Lagalante and Greenbacker 2007). In this study, a 100 µl negative control was used, and each calibrator (0.2, 1, 5, and 6 ppb), and xylem samples were added in duplicate to individual wells. To each well 100 µl of the enzyme (HRP)-labeled imidacloprid was then added. The plate was covered with a sheet of Parafilm and shaken at 200 rpm on an orbital plate shaker. After 1 h, the well contents were emptied, vigorously rinsed with cool tap water, and the well-plate was slapped on a paper towel to remove all visible water. When the plate was dry, 100 µl of substrate (hydrogen peroxide) was added to each well. The kit was covered with a new sheet of Parafilm and shaken at 200 rpm on an orbital plate shaker. After 30 min, 100 µl of a stop solution (1.0 M HCl) was added to each well and the optical density was read at 450 nm (600 nm reference wavelength) using a Bio-Rad Benchmark Plus (Hercules, CA) plate reader at 25 °C (Jones 2007). The greater the amount of imidacloprid bound in a well, the less the optical density. A negative control is used to calculate B_0, which is the amount of HRP bound in the absence of imidacloprid. The % B_0 is the ratio of the optical density of each of the samples to the optical density of the negative control times 100 (Cook 2008).

The Envirologix ELISA kits used to detect imidacloprid do not distinguish between imidacloprid, its metabolites, and other chemical compounds containing similar chemical groups (Lagalante and Greenbacker 2007). To account for this effect of using ELISA on natural matrices, xylem fluid was collected from untreated trees. These samples were prepared for analysis at the following dilutions: undiluted, 10-, 20-, 50-, and 100-fold dilutions. The results of this calibration showed that a 20-fold dilution produced an optical density that was equivalent to the negative controls (Jones 2007). However, a 20-fold dilution of hemlock xylem fluid elevated the working range and limit of detection of the ELISA kit from 0.2–6 ppb to 4–120 ppb. If the measured imidacloprid concentration of xylem fluid sample was higher than 120 ppb, further dilutions were made (e.g., 1:10, 1:100, or 1:1,000) to bring the sample into the working range of the ELISA kit (Cowles et al. 2006, Jones 2007, Eisenback et al. 2009).

As a comparison to the ELISA samples, a random subset of 125 samples was analyzed using derivatization GC/MS. Concentrations of imidacloprid as the heptafluorobutyric derivative were determined on a Star software (Varian, Walnut Creek, California) computer-controlled Varian 3900 gas chromatograph. The Varian 1177 injector was fitted with a Merlin Microseal septum. The injector temperature was maintained at 250 °C and a splitless injection was used. Separation was accomplished using a Varian VF-5 MS column (30 m, 0.26 mm i.d., 0.25 µm phase thickness). The column temperature program was 80 °C (2-min hold) to 250 °C at 20 °C/min then to 320 °C at 10 °C/min (0.5-min hold). The helium carrier gas was electronic pressure controlled at a constant flow of 1.0 ml/min. The Varian 2100T ion-trap mass spectrometer was operated in CI+ mode (acetonitrile liquid CI reagent, multiplier 1,400 V, *m/z* range of 50–450) (Jones 2007).

Imidacloprid Concentration in Leaf Tissue

To determine the concentration of imidacloprid in leaf tissue, a subsample of three trees from each of the two injection methods were selected from the spring treatment at Site B. Needles were removed from the same branches used for xylem fluid analysis. The twigs were separated by new growth and old growth (based on position) and placed in separate paper bags. The bagged samples were then air dried overnight, and dried at 60 °C for a minimum of 4 h in a drying oven. Once dried, the needles were separated from twigs, and pulverized using a coffee grinder (Mr. Coffee, Model IDS55, Cleveland, Ohio) (Cowles et al. 2006), then placed in opaque storage containers and frozen at −18 °C. A 1:10 (needle: solvent) ratio was used to extract the compounds from the hemlock needles because this ratio was known to be adequate for needle extraction (Cowles et al. 2006). A total of 1.5 ml of extraction solvent was added to 0.15-g dried needles in a 2.0-ml microcentrifuge tube (Fischer Scientific, Hampton, New Hampshire). The microcentrifuge tubes were shaken overnight on an orbital bench shaker (Model G33, New Brunswick Scientific, Edison, New Jersey). The microcentrifuge tubes were then spun down on a Heraeus Instruments benchtop microcentrifuge (Biofuge 13, Heraeus Instruments, Germany) at 13,000 G for 10 min. The supernatant extraction solvent was removed by pipette and transferred to an autosampler for LC/MS/MS analysis (Cook 2008).

Statistical Analysis

Imidacloprid concentration data were analyzed using a generalized linear mixed-effects model using PROC MIXED (SAS Institute 2011). Individual trees were considered as the experimental unit with site as a blocking variable and concentration of imidacloprid as the response variable. Tests for significance for the factors of site, application method, treatment season, height sections, quadrant, and weeks posttreatment as well as the random effects of height section, quadrant and week posttreatment (nested within tree) along with each two-way interaction were tested using type III *F*-ratios. The model used an unstructured covariance model. A total of 90 ELISA observations were classed as outliers using studentized residuals (±3 SD from the mean) and excluded from the analysis. The conventional $\alpha = 0.05$ level of significance was used to determine variable retention in the model. Site and quadrant were found to be nonsignificant and were removed from the model along with all nonsignificant two-way interactions. Multiple comparisons of means were conducted using Tukey-Kramer tests. We reported adjusted *P* values that could be interpreted in a fashion similar to an experiment-wise error rate of $\alpha = 0.05$.

The association between GC/MS and ELISA was investigated by computing Pearson's correlation coefficient (r) and regression analysis using PROC CORR and PROC REG (SAS Institute 2011), respectively. The concentrations of imidacloprid in xylem fluid analyzed by both ELISA and GC/MS were not normally distributed and

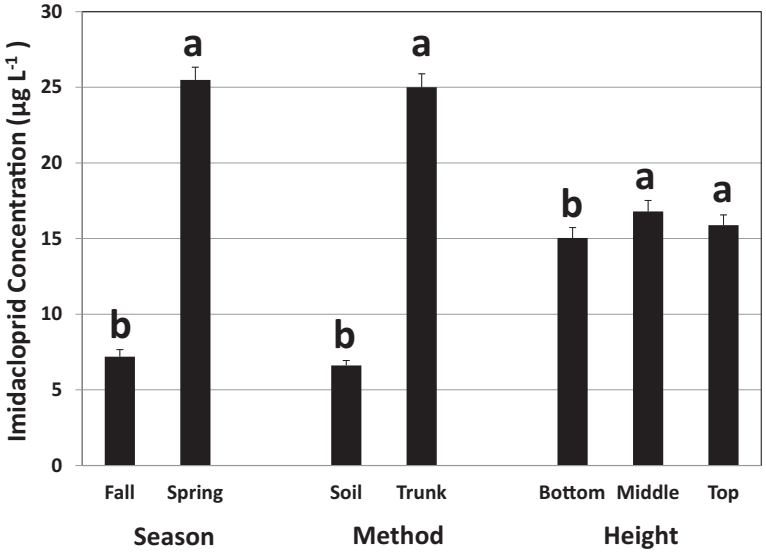

Fig. 1. Imidacloprid xylem concentrations (mean ± SEM), determined by ELISA, by treatment season, treatment method and height section for treated eastern hemlock. Means sharing a letter in each category (i.e., season, method, and height) are not significantly different at the 0.05 level according to Tukey-Kramer multiple comparisons tests.

consequently both were transformed using the natural logarithm (ln) of concentration. Seventeen observations were classed as outliers (±3 SD from the mean) and excluded from the analysis. The association between ELISA and LC/MS/MS was investigated using PROC CORR (SAS Institute 2011). Because of the skewed data distributions with numerous zero values of both the ELISA and LC/MS/MS data, which violated the normality assumptions needed for Pearson correlation, Spearman's rank correlation was used. Based on the results of the ELISA and GC/MS comparison we chose to use a binary response variable (0/1), detected or undetected, to compare the imidacloprid levels in xylem fluid to the imidacloprid in leaf tissue found within the same branch. These data were analyzed using the continuity adjusted chi-square test in PROC FREQ (SAS Institute 2011).

Results

Spatial and Temporal Distribution of Imidacloprid in Xylem Fluid

Xylem fluid concentrations of imidacloprid extracted from branch samples within trees were highly variable. Of the 3,475 xylem samples analyzed by ELISA, only 1,494 samples (43%) were positive for imidacloprid. Of the 64 trees treated in this project, 63 (98%) had detectable levels of imidacloprid in at least one sample of xylem fluid; only one fall soil-injected tree never showed detectable levels of imidacloprid. Significant differences in imidacloprid concentration in xylem fluid were found between treatment season (F = 158.24; df = 1, 2941; $P < 0.0001$), application method ($F = 46.31$; df = 1, 2941; $P < 0.0001$), height ($F = 4.98$; df = 2, 187; $P < 0.01$; Fig. 1), and weeks posttreatment ($F = 42.5$; df = 4, 248; $P <$ 0.001). None of the two-way interactions were significant. Xylem fluid concentrations were significantly higher (posthoc Tukey–Kramer test: $t = -12.58$, df = 2,941; $P < 0.0001$) for spring than fall applications with averages of 25.49 and 7.19 µg/l (ppb), respectively. The trunk injection application method produced significantly higher ($t = -6.80$, df = 2941; $P < 0.0001$) concentrations of imidacloprid in xylem fluid than soil injection with averages of 25.00 and 6.61 µg/l, respectively. Mean concentration of imidacloprid was significantly lower in the bottom section of the tree crown than either the middle ($t = -2.79$, df = 187; $P = 0.016$) or top ($t = -2.69$, df = 187; $P = 0.021$) sections; no statistically significant difference was found between the middle and top sections ($t = 0.10$; df = 187; $P = 0.995$) across all application methods and seasons. Detectable concentrations of imidacloprid were found in xylem fluid 3 weeks posttreatment with concentrations increasing over the weeks with the highest concentration found at 52 weeks posttreatment. Differences in mean concentration levels began to appear at week 3 with statistically significant difference documented between weeks 3 and 52 ($t = -11.47$; df = 248; $P < 0.0001$), weeks 9 and 52 ($t = -9.79$; df = 248; $P < 0.0001$), weeks 15 and 52 ($t = -10.33$; df = 248; $P < 0.0001$), and weeks 21 and 52 ($t = -9.28$; df = 248; $P < 0.0001$; Table 1).

Table 1. Mean imidacloprid concentration (ppb) in xylem fluid, determined by ELISA from eastern hemlock

Weeks posttreatment	Imidacloprid concentration (ppb) ± SD
3	8.08 ± 25.75^{a}
9	12.72 ± 33.03^{ab}
15	12.98 ± 34.04^{abc}
21	16.2 ± 38.37^{abc}
52	29.25 ± 37.06^{d}

Means sharing a letter in the superscript are not significantly different at the 0.05 level according to Tukey-Kramer multiple comparisons tests.

A moderate positive correlation ($n = 107$; $r = 0.678$; $P <$ 0.0001) was found between ELISA and GC/MS imidacloprid concentrations as determined by each method (O'Rourke et al. 2005). The linear regression for imidacloprid concentration between GC/MS and ELISA was $y = 0.56\,x + 1.62$, where x is the natural log value of imidacloprid concentration determined by ELISA and y is the natural log value of imidacloprid concentration determined by GC/MS ($F = 89.18$; df = 1, 105; $P < 0.0001$; $r^2 = 0.459$) (Fig. 2). For the 106 samples examined, 69% of the samples analyzed by ELISA give higher concentrations of imidacloprid than those found by GC/MS, leading to evidence of a significant matrix effect and overestimation of imidacloprid in xylem fluid by ELISA. GC/MS

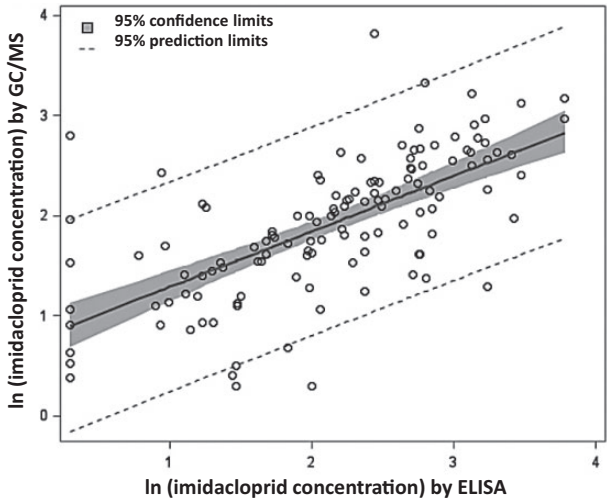

Fig. 2. Comparison for imidacloprid in xylem fluid samples between ELISA and GC/MS. The regression equation was $y = 0.56\,x + 1.62$, where x is the *ln* (imidacloprid concentration) determined by ELISA and y is the *ln* (imidacloprid concentration), and the regression coefficient: $R^2 = 0.459$.

detected imidacloprid in all 106 samples analyzed as where ELISA detected imidacloprid in 100 (94%) of the samples analyzed.

Imidacloprid Concentration in Leaf Tissue

A significant positive correlation was found between the levels of imidacloprid in the xylem fluid compared with the levels in leaf tissue ($n = 235$; $r = 0.36$; $P < 0.0001$). A statistically significant difference in imidacloprid concentration was found between xylem fluid (ELISA) and leaf samples (LC/MS, $\chi^2 = 14.17$, df $= 1$, $P < 0.001$). Of 235 samples analyzed, 36% (84 samples) had no detectable imidacloprid in either the xylem fluid or leaf samples. Detectable levels of imidacloprid were found in both xylem and leaf samples 27% (63 samples) of the time. The remaining samples had mixed results, with 14% (34 samples) of the samples having detectable imidacloprid in the xylem fluid but not in the leaf samples, and 23% of the leaf samples having detectable imidacloprid did not show detectable levels in their xylem tissue.

Discussion

Previous imidacloprid efficacy tests conducted with *A. tsugae*-infested trees have shown significant differences in imidacloprid concentration between treatment methods (i.e., soil injection and truck injection) and within the crown of *A. tsugae*-infested eastern hemlock using ELISA (Cowles et al. 2006, Dilling et al. 2010). In our study, trees with similar sized crowns without *A. tsugae* were used, thus allowing us to look at the spatial and temporal distribution of imidacloprid without any confounding issues related to *A. tsugae* feeding, crown size and tree response. The results of our study showed that ELISA detected differences by season, application method, height, and weeks posttreatment; however, no significant difference for site, direction, or any two-way interactions were detected. Imidacloprid concentrations detected within xylem fluid were very similar to those found in other studies that used infested trees (Cowles et al. 2006, Dilling et al. 2010). In laboratory dose-response tests from the previous studies, the LC_{50} of imidacloprid was reported as 122, 300, and 242 ppb at 15, 20, and 30 day exposures, respectively (Cowles et al. 2006, Eisenback et al 2010, Coots 2012). All the observed averages of imidacloprid found in this study (Fig. 1, Table 1) were smaller than the LC_{50} reported for *A. tsugae*.

The LCR is the ratio of crown length to tree height (Olivier and Larson 1996) and is a measure of a tree's foliar canopy. Although standardized in this study, no mention of crown size occurs in the previous studies of imidacloprid movement in eastern hemlock (Tattar et al. 1998, Cowles et al. 2006, Dilling et al. 2010). Although our results are similar to those of other studies, it does raise the question of how the results of previous studies on the spatial and temporal distribution of imidacloprid were impacted by the crown size and presence and spatial arrangement of *A. tsugae*.

ELISA is a popular tool for imidacloprid quantification, but it also can produce false positives and overestimate imidacloprid concentrations due to matrix effects in sap (Cowles et al. 2006), needle tissue, and wood (Eisenback et al. 2009). A matrix effect occurs when nontarget compounds in a sample bind with ELISA antibodies (Sullivan and Goh 2000, Byrne et al. 2005). This nonspecific binding can cause a false positive response and overestimation of imidacloprid concentration in samples (Cowles et al. 2006, Eisenback et al. 2009). To account for this effect samples must be diluted. Although the dilution decreases the detection limit of imidacloprid by ELISA, it still allows the sensitivity of the kit to be within the biological efficacy against *A. tsugae* (Cowles et al. 2006, Jones 2007, Eisenback et al. 2009). In this study, a 20-fold dilution was used to account for this effect in xylem fluid (Eisenback et al. 2009), but the dilution might not be sufficient to account for all the potential individual tree and seasonal effects of metabolism on imidacloprid and its metabolites within the tissue of eastern hemlock. In addition to ELISA, other detection methods that did not suffer from a matrix effect were used, allowing us to investigate the movement of imidacloprid within xylem fluid (ELISA) and leaf tissue (LC/MS/MS) (Cook 2008, Eisenback et al. 2009). In nearly a quarter of the samples analyzed by both ELISA and LC/MS/MS, imidacloprid was found in the needles but not in the xylem fluid of individual branches. This point to several possibilities, two of which may be that imidacloprid was present in the xylem fluid but was below the detection of the ELISA kit, or that imidacloprid is moving intermittently within the crown and was not present at the day and time the branch was collected. Cowles et al. (2006) found concentrations of imidacloprid in new growth tissue similar to that of previous year's growth, and suggested that either remobilization or continued uptake was occurring after application.

Imidacloprid is a water soluble insecticide and is believed to move by mass flow in the transpiration stream (water flux) (Vité and Rudinsky 1959, Ford et al. 2010) of eastern hemlock. This movement and distribution could be affected by numerous factors, such as the availability of water, the season, the time of day, tree condition, tree size, the amount of crown, or the level of *A. tsugae* infestation (Ford et al. 2007). Numerous factors could be affecting the movement and distribution of imidacloprid, some of these factors, such as the availability of water, season, time of day, tree condition, tree size, amount of crown, infestation levels, and local environmental factors (Ford et al. 2007).

Imidacloprid has been shown to be an effective insecticide against *A. tsugae* regardless of season and treatment method (McAvoy et al. 2005, Cowles et al. 2006, Doccola et al. 2007). Although site-specific (e.g., soil type) and tree-specific factors (e.g., amount of new growth, current tree condition, *A. tsugae* density, and LCR) must be taken into account when choosing the method, dosage, and season of treatment, all of these factors are likely to affect an individual tree's ability to transport these insecticides and provide effective control of *A. tsugae*. This suggests that

pretreatment of eastern hemlocks at high risk from *A. tsugae* can be justified, if only to allow for better spatial distribution and movement of imidacloprid within the crown of uninfested hemlock trees.

Currently the amount of systemic insecticide applied is based on tree dbh (Steward and Horner 1994, Fidgen et al. 2002, Silcox 2002, Doccola et al. 2007), with no change in dosage for differences in crown volume. Most recently, xylem water movement models (Ford et al. 2010) have been developed for eastern hemlock that show water usage (mass flow) is exponentially related to tree diameter, with smaller trees using proportionally less water than larger trees. This work has shown that the current manufacturer's recommended dose, which is based on a linear function of tree diameter, can be scaled to match crown volume and still provide similar amounts of imidacloprid. The next step in this progression is to develop models that account for crown volume differences (Turcotte et al. 2008b). Future research is needed to develop crown volume equations that could be used as the foundation for the development of new treatment tables based not only on tree diameter but also on the amount of live crown present (e.g., a tree with 30-cm dbh and 80% LCR vs. a tree with 30-cm dbh and 40% LCR). This could reduce the amount of imidacloprid used and cost of treating *A. tsugae*-infested eastern hemlock.

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References Cited

Byrne, F. J., S. J. Castle, J. L. Bi, and N. C. Toscano. 2005. Application of competitive enzyme-linked immunosorbent assay for the quantification of imidacloprid titers in xylem fluid extracted from grapevines. J. Econ. Entomol. 98: 182–187.

Costa, S. D. 2005. Sampling for detection and monitoring of hemlock woolly adelgid within hemlock stands. Proceedings, pp. 57–62. *In* B. Onken and R. Reardon (eds.), Third symposium on hemlock woolly adelgid in the Eastern United States; Asheville, NC. Department of Agriculture, Forest Service, Forest Health Technology Enterprise Team, Morgantown, WV.

Costa, S., and B. Onken. 2006. Standardizing sampling for detection and monitoring of hemlock woolly adelgid in eastern hemlock forests. Forest Health Technology Enterprise Team, FHTET-2016-16, Morgantown, WV.

Cheah, C., M. E. Montgomery, S. M. Salom, B. L. Parker, S. Costa, and M. Skinner. 2004. Biological Control of Hemlock Woolly Adelgid. USFS FHTET Report, FHTET-2004-04.

Cook, B. F. 2008. Liquid chromatography tandem mass spectrometry (LC/MS/MS) determination of the uptake, persistence and metabolism of imidacloprid in treated hemlock trees. M. Sc. thesis, Villanova University, Villanova, PA, 68 p.

Coots, C. I. 2012. Spatial, temporal, and tri-trophic distribution of imidacloprid, olefin and 5-Hydroxy and their effect on hemlock woolly adelgid, Adelges tsugae Annand (Hemiptera: Adelgidae). Ph.D. Dissertation, University of Tennessee, Knoxville TN. 133 p.

Cowles, R. S., M. E. Montgomery, and C. A. S. J. Cheah. 2006. Activity and residues of Imidacloprid applied to soil and tree trunk to control hemlock woolly adelgid (Hemiptera: Adelgidae) in forests. J. Econ. Entomol. 99: 1258–1267.

Di Muccio, A., P. Fidente, D. A. Barbarini, R. Dommarco, S. Seccia, and P. Morrica. 2006. Application of solid-phase extraction and liquid chromatography-mass spectrometry to the determination of neonicotinoid pesticide residues in fruit and vegetables. J. Chromat. 1108: 1–6.

Dilling, C., P. Lambdin, J. Grant, and R. Rhea. 2010. Spatial and temporal distribution of Imidacloprid in in eastern hemlock in the southern Appalachians. Ecol. Entomol. 103: 368–373.

Doccola, J. J., E. J. Bristol, S. D. Sifleet, J. Lojiko, and P. M. Wild. 2007. Efficacy and duration of trunk-injected imidacloprid in the management of Hemlock woolly adelgid (*Adelges tsugae*). Arbor. Urban For. 33: 2–21.

Eisenback, B. M., D. E. Mullins, S. M. Salom, and L. T. Kok. 2009. Evaluation of ELISA for imidacloprid detection in eastern hemlock (*Tsuga canadensis*) wood and needle tissue. Pest Manag. Sci. 65: 122–128.

Eisenback, B. M., S. M. Salom, L. T. Kok, and A. F. Lagalante. 2010. Lethal and sublethal effects of imidacloprid on hemlock woolly adelgid (*Adelges tsugae*, Hempitera: Adelgidae) and two introduced predator species in the laboratory. Econ. Entomol. 103: 1222–1234.

EnviroLogic. 2004. QuantiPlate Kit for Imidacloprid. (http://www.envirologix.com/library/ep006insert.pdf) (accessed 19 May 2016).

Evans, A. M., and T. G. Gregoire. 2007. The tree crown distribution of hemlock woolly adelgid, *Adelges tsugae* (Hem., Adelgidae) from randomized branch sampling. J. Appl. Entomol. 131: 26–33.

Fidgen, J. G., Q. C. McClellan, and S. M. Salom. 2002. Efficacy and residual activity of two systemic insecticides for control of hemlock woolly adelgid on young eastern hemlocks, pp. 329. *In* B. Onken, R. Reardon, and J. Lashomb (eds.), Proceedings Hemlock Woolly Adelgid in the Eastern United States. NJ Agricultural Experiment Station, New Brunswick, New Jersey.

Ford, C. R., B. C. Reynolds, and J. M. Vose. 2010. Xylem transport models optimize effectiveness of systemic insecticide applications for controlling hemlock woolly adelgid (*Adelges tsugae*). US Department of Agriculture, Forest Service, Southern Research Station.

Ford, C. R., J. M. Vose, M. Daley, and N. Phillips. 2007. Use of water by eastern hemlock: implications for systemic insecticide application. Arbor. Urban for. 33: 421–427.

Haukioja, E. 1991. Induction of defenses in trees. Annu. Rev. Entomol. 36: 25–42.

Jeschke, P., R. Nauen, M. Schindler, and A. Elbert. 2010. Overview of the status and global strategy for neonicotinoids. J. Agric. Food Chem. 59: 2897–2908.

Jeschke, P., and R. Nauen. 2008. Neonicotinoids—from zero to hero in insecticide chemistry. Pest Manag. Sci. 64: 1084–1098.

Jones, J. 2007. Determination of imidacloprid by ELISA and GC/MS: A comparison of analytical techniques and a coordinated field study with the U.S. Forest Service to determine uptake and persistence in imidacloprid treated hemlock trees. M.Sc. thesis, Villanova University, Villanova, PA, 69 p.

Karban, R., and I. T. Baldwin. 2007. Induced responses to herbivory. University of Chicago Press. 319 pp.

Knauer, K., J. Linnane, K. Sheilds, and R. Bridges. 2002. An initiative for management of hemlock woolly adelgid, pp. 9-11. *In* B. Onken, R. Reardon, and J. Lashomb (eds.), Proceedings Hemlock Woolly Adelgid in the Eastern United States. NJ Agricultural Experiment Station, New Brunswick, New Jersey.

Lagalante, A. F., and P. W. Greenbacker. 2007. Flow injection analysis of imidacloprid in natural waters and agricultural matrixes by photochemical dissociation, chemical reduction, and nitric oxide chemiluminescence detection. Analyt. Chim. Acta. 590: 151–158.

Li, K., and Q. X. Li. 2000. Development of an enzyme-linked immunosorbent assay for the insecticide imidacloprid. J. Agric. Food Chem. 48: 3378–3382.

McDonald, L. M., and T. R. Meyer. 1998. Determination of imidacloprid and triadimefon in white pine by gas chromatography/mass spectrometry. J. Agric. Food Chem. 46: 3133–3138.

McAvoy, T., W. T. Mays, S. M. Salom, and L. T. Kok. 2005. Impact of imidacloprid on hemlock woolly adelgid and water quality at Mt. Lake, Virginia, pp. 324–334. *In* B. Onken and R. Reardon (eds.), Proceedings of the Third Symposium on the Hemlock Woolly Adelgid in the Eastern United States. USDA Forest Service, Forest Health Technology Enterprise Team 2005-01, Morgantown, West Virginia.

McClure, M. S. 1989. Evidence of a polymorphic life cycle in the hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae). Ann. Entomol. Soc. Am. 82: 50–54.

McClure, M. S. 1991. Density-dependent feedback and population cycles in *Adelges tsugae* (Homoptera: Adelgidae) on *Tsuga canadensis*. Environ. Entomol. 20: 258–264.

McClure, M. S. 1995. Managing hemlock woolly adelgid in ornamental landscapes. Bulletin 925. Connecticut Agricultural Experiment Station, 7 p.

McClure, M. S. 2001. Biological control of hemlock woolly adelgid in the Eastern United States. USDA, Forest Service, Forest Health Technology Enterprise Team, Morgantown, WV, FHTET-2000-08, 10 p.

Mullins, J. W. 1993. Imidacloprid: a new nitroguanidine insecticide, pp 183–189. *In* S. O. Duke, J. J. Menn, and J. R. Plimmer (eds.), Pest control with enhanced environmental safety by American Chemical Society Symposium, ACS, Washington, DC.

Nykänen, H., and J. Koricheva. 2004. Damage-induced changes in woody plants and their effects on insect herbivore performance: a meta-analysis. Oikos 104: 247–268.

Olivier, C. D., and B. C. Larson. 1996. Forest stand dynamics. McGraw-Hill, New York, 520 p.

O'Rourke, N., L. Hatcher, and E. J. Stepanski. 2005. A step-by-step approach to using SAS for univariate & multivariate statistics. SAS Institute, Wiley, New York, 237 p.

Radville, L., C. Arielle, and E. L. Preisser. 2011. Variation in plant defense against invasive herbivores: evidence for a hypersensitive response in eastern hemlocks (*Tsuga canadensis*). J. Chem. Ecol. 37: 592–597.

Rouchaud, J., F. Gustin, and A. Wauters. 1996. Imidacloprid Insecticide soil metabolism in sugar beet field crops. Bull. Environ. Contam. Toxicol. 53: 29–36.

SAS Institute. 2011. SAS User guide version 9.4. SAS Institute, Cary, NC.

Schroeder, M. E., and R. F. Flattum. 1984. The mode of action and neurotoxic properties of the nitromethylene heterocycle insecticides. Pestic. Biochem. Physiol. 22: 148–160.

Silcox, C. A. 2002. Using imidacloprid to control hemlock woolly adelgid, pp. 280–287. *In* B. Onken and R. Reardon (eds.), Proceedings Hemlock Woolly Adelgid in the Eastern United States. NJ Agricultural Experiment Station, New Brunswick, New Jersey.

Smith, K. T., and P. A. Lewis. 2005. Potential concerns for tree wound response from stem injections, pp. 173–178. *In* B. Onken and R. Reardon (eds.), Proceedings of the Third Symposium on the Hemlock Woolly Adelgid in the Eastern United States. USDA Forest Service, Forest Health Technology Enterprise Team 2005-01, Morgantown, West Virginia.

Smith, S. F., and V. A. Krischik. 1999. Effects of systemic imidacloprid on *Coleomegilla maculata* (Coleoptera: Coccinellidae). Environ. Entomol. 28: 1189–1195.

Steward, V. B., and T. A. Horner. 1994. Control of hemlock wooly adelgid using soil injections of systemic insecticides. J. Arbor. 20: 287–289.

Sullivan, J. J., and K. S. Goh. 2000. Evaluation and validation of a commercial ELISA kit for diazinon in surface waters. J. Agric. Food Chem. 48: 4071–4078.

Tattar, T. A., J. A. Dotson, M. A. Ruizzo, and V. B. Steward. 1998. Translocation of imidacloprid in three tree species. J. Arbor. 24: 54–56.

Turcotte, R. M., L. M. McDonald, and K. B. Piatek. 2008a. Spatial distribution of fine roots and soil carbon beneath eastern hemlock, pp. 269. *In* B. Onken and R. Reardon (eds.), Proceedings of the Third Symposium on the Hemlock Woolly Adelgid in the Eastern United States. USDA Forest Service, Forest Health Technology Enterprise Team 2008-01, Morgantown, West Virginia.

Turcotte, R. M., J. R. Brooks, and A. Cumpston. 2008b. Improving the accuracy of crown volume estimates in eastern hemlock, pp. 267–268. *In* B. Onken and R. Reardon (eds.), Proceedings of the Third Symposium on the Hemlock Woolly Adelgid in the Eastern United States. USDA Forest Service, Forest Health Technology Enterprise Team 2008-01, Morgantown, West Virginia.

Turcotte, R. M. 2016. Temporal and spatial distribution of imidacloprid and the arthropod fauna associated with eastern hemlock, Tsuga canadensis (L.) Carr. Ph.D. Dissertation. West Virginia University, Morgantown WV. 112 p.

[USDA] United States Department of Agriculture. 2014. HWA distribution map. (http://http://na.fs.fed.us/fhp/hwa/maps/2014.pdf).

Vité, J. P., and J. A. Rudinsky. 1959. The water-conducting systems in conifers and their importance to the distribution of trunk injected chemicals. Contr. Boyce Thomps. Inst. 20: 27–38.

Young, R. F., K. S. Shields, and G. P. Berlyn. 1995. Hemlock woolly adelgid (Homoptera: Adelgidae): Stylet bundle insertion and feeding sites. Ann. Entomol. Soc. Am. 88: 827–835.