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BENEFITS AND RISKS OF IMIDACLOPRID-BASED MANAGEMENT PROGRAMS FOR HEMLOCK WOOLLY ADELGID

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I am submitting herewith a dissertation written by Elizabeth Paige Benton entitled "BENEFITS AND RISKS OF IMIDACLOPRID-BASED MANAGEMENT PROGRAMS FOR HEMLOCK WOOLLY ADELGID." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Entomology and Plant Pathology.

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BENEFITS AND RISKS OF IMIDACLOPRID-BASED MANAGEMENT PROGRAMS FOR HEMLOCK WOOLLY ADELGID

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DEDICATION

I dedicate this dissertation to my family: Dan, Violet, and Russ Benton. This endeavor would not have been possible without their love, support, and sacrifice.

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ABSTRACT

Hemlock woolly adelgid, *Adelges tsugae* (Annand) (HWA) (Hemiptera: Adelgidae), has caused widespread decline of eastern hemlock, *Tsuga canadensis* (L.) Carriére. This collaborative retrospective analysis with Great Smoky Mountains National Park (GRSM) provides research-based management guidance on imidacloprid treatments and nontarget risks to aquatic systems.

Imidacloprid and olefin concentrations were assessed in foliage from different diameter at breast height (DBH) size hemlocks 4 – 7 yr post-imidacloprid treatment. Imidacloprid concentrations were below the LC_{50} [lethal concentration] for HWA, but olefin was above the LC_{50} 4 yr post-treatment. HWA populations were suppressed, and hemlock canopies were healthy. Treatment efficacy can last up to 7 yr post-treatment.

Hemlocks from the larger (61 and 76 cm) size classes generally had higher concentrations of imidacloprid and olefin. Concentration data from foliage of different size hemlocks were used to develop a model to optimize dosages based on tree diameter. Smaller (< 30 cm) and larger (> 63 cm) hemlocks can be given lower imidacloprid doses, while maintaining > 80% HWA mortality in hemlocks.

Impacts to stream water quality and aquatic macroinvertebrates were assessed in ten streams in hemlock conservation areas. Water samples were collected upstream and downstream from conservation areas and in nearby control streams. Imidacloprid was present in seven downstream locations in concentrations from 28.5 to 379 ppt, which is below USEPA aquatic life benchmarks. Aquatic macroinvertebrate bioassessments were conducted at downstream and

upstream locations, and downstream baseline data were available. Diversity measures at downstream samples did not vary from those at upstream and baseline samples. Imidacloprid treatments did not negatively affect aquatic macroinvertebrate communities.

Imidacloprid treatments suppress HWA populations for up to 7 yr. Dosages can be optimized based on the DBH size of the hemlock. Imidacloprid risks to aquatic systems for this HWA management program were minimal and within USEPA benchmarks.

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CHAPTER I. INTRODUCTION

Eastern Hemlock

Natural habitats are currently experiencing a large amount of pressure from exotic and invasive pest species (Vitousek et al. 1996, 1997, Mack et al. 2000). The intrusion of exotic species in a habitat can cause structural and functional changes to occur in natural systems (Castello et al. 1995, Liebhold et al. 1995, Ellison et al. 2005). This phenomenon is not only an ecological process, but an economic one as well (Liebhold et al. 1995). Every year in the United States exotic species in forests cause more than two billion dollars in economic impacts due to control expenses and product losses (Pimentel et al. 2000, Aukema et al. 2011). In the last 100 years, many tree species in eastern forests of the United States have been negatively affected by the feeding activity or pathogen transmission by nonnative insect pests. The following trees have either been lost or have suffered a massive decline in abundance: American beech (*Fagus grandifolia* Ehrh.), American chestnut (*Castanea dentate* [Marsh.] Borkh.), eastern hemlock (*Tsuga canadensis* [L.] Carriére), Carolina hemlock (*Tsuga caroliniana* Engelm.), elm (*Ulmus* spp.), and oak (*Quercus* spp.) (Liebhold et al. 1995).

Eastern hemlock populations have been in decline due to hemlock woolly adelgid, *Adelges tsugae* (Annand) (HWA) (Hemiptera: Adelgidae). The range of eastern hemlock spans much of the eastern United States (Figure 1) (USGS 1999). Hemlock is distributed as far south as northern Alabama and Georgia north to New England, and east to the Atlantic coast and as far west as Wisconsin (USDA Forest Service 1990). Eastern hemlock has a profound influence on the structure and function of its surrounding ecosystem and is an important foundation species in southern Appalachian forests (Ellison et al. 2005). Hemlock is a slow-growing species that

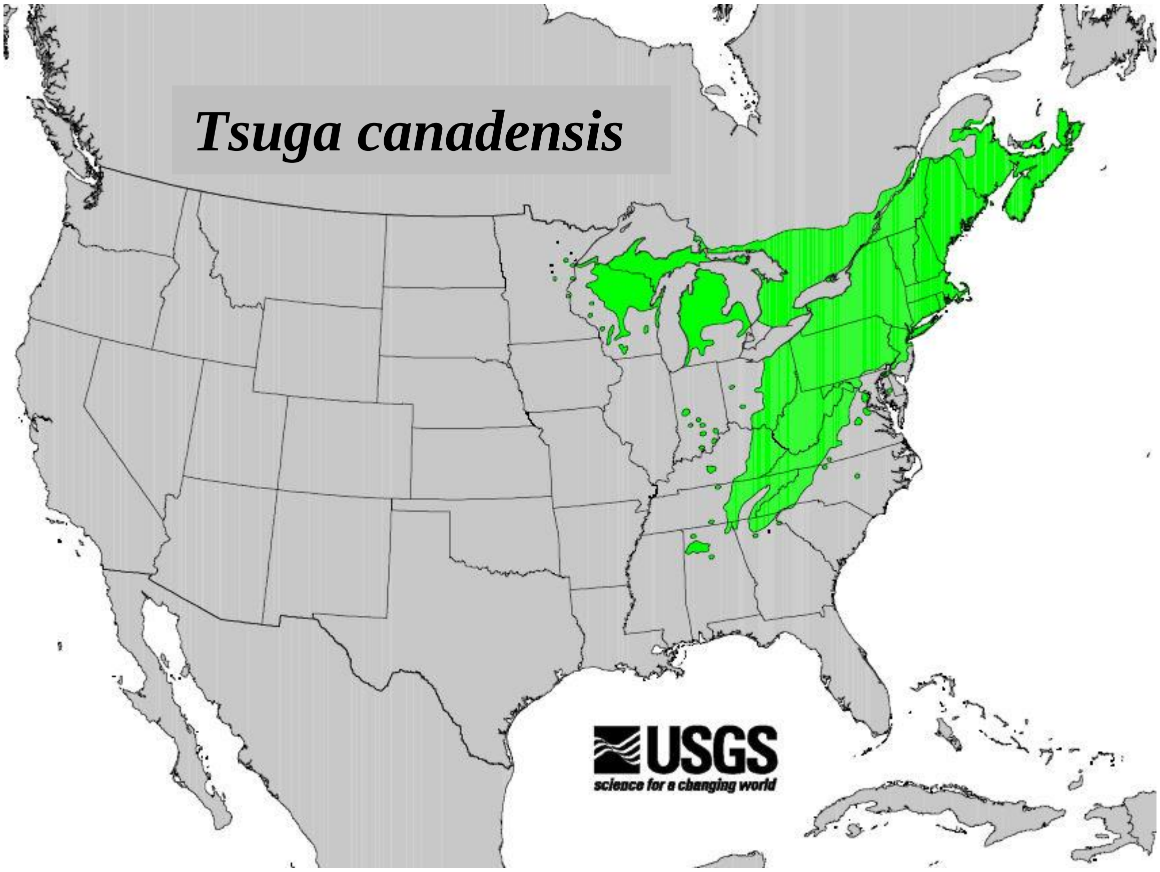


Figure 1. Native range of eastern hemlock (USGS 2013).

inhabits a distinctive ecological niche (Orwig and Foster 1998, Ward et al. 2004). Unfortunately, successful regeneration under continued HWA pressure would be difficult, as hemlock cannot re-foliate and must be propagated by seeds which are only viable for up to 4 yr (Olson et al. 1959, Orwig and Foster 1998).

As the only shade-tolerant conifer, eastern hemlock plays a vital ecological role in forests, and that role cannot be filled by any other native evergreen tree species (Orwig and Foster 1998, Ward et al. 2004). As a low-growing evergreen, hemlock foliage is a source of food for deer in the winter (Lapin 1994). Hemlock provides habitat for snowshoe rabbit (*Lepus americanus*), turkey (*Meleagris gallopavo)*, ruffed grouse (*Bonasa umbellus*), and numerous other vertebrate species (Jordan and Sharp 1967), including many other bird species (Tingley et al. 2002). Eastern hemlock systems have diverse arthropod communities, with greater than 400 insect species associated with hemlocks (Wallace and Hain 2000, Buck et al. 2005, Lynch et al. 2006, Dilling et al. 2007, 2009, Coots et al. 2012). Both Hakeem (2008) and Mallis and Rieske (2011) also found rich arthropod predator communities associated with eastern hemlock. Soil arthropods, especially the collembolans, associated with eastern hemlock are diverse (Reynolds 2008).

In addition, hemlocks provide leaf and coarse woody debris inputs into streams and function to provide stream systems with deep shade, resulting in a cool microclimate (Rogers 1978, Hadley 2000, Huddleston 2011). Hemlocks transpire year round, which regulates stream discharge rates in the winter, resulting in stabilizing seasonal streamflow (Ford and Vose 2007). Streams associated with hemlock have higher aquatic macroinvertebrate species richness and higher fish trophic diversity compared to streams in hardwood-dominated watersheds (Snyder et

al. 2002, Ross et al. 2003, Webster et al. 2012). Since many species are dependent on this foundation species, continued cascading terrestrial and aquatic ecosystem impacts are expected in eastern forests with continued hemlock decline.

Carolina hemlock, a species similar to eastern hemlock, with a limited distribution, is vulnerable to decline due to HWA (Figure 2). This species is endemic to the upper Piedmont and southern Appalachians in the eastern United States (Harlow et al. 1996) and exists in relict populations on ridge tops and bluffs (Rentch et al. 2000). Carolina hemlock is a late successional species (Colandonato 1993) and is listed on Tennessee's Natural Heritage Program's Rare Plant List (2008).

Hemlock Woolly Adelgid

HWA is an invasive insect with genetically differentiated populations in Japan, China, and the Pacific Northwest of the United States (Havill et al. 2006). Although first documented in the United States on western hemlock (*Tsuga heterophylla* [Raf.] Sarg.) (Pinales: Pinaceae) as early as 1922 in British Columbia and soon after in California and Oregon (Annand 1926), western hemlock appears to tolerate feeding. HWA was later documented in the eastern United States from Virginia in 1951 (Stoetzel 2002) with a population later identified as having originated in Japan (Havill et al. 2006).

The native hosts of HWA in Japan are Northern Japanese hemlock (*Tsuga diversifolia* Masters) and Southern Japanese hemlock (*T. sieboldii* Carriére) (McClure 1995). While there are nine *Tsuga* species worldwide, HWA is only a serious pest of eastern hemlock and Carolina hemlock, the species native to the eastern United States. It appears that neither species

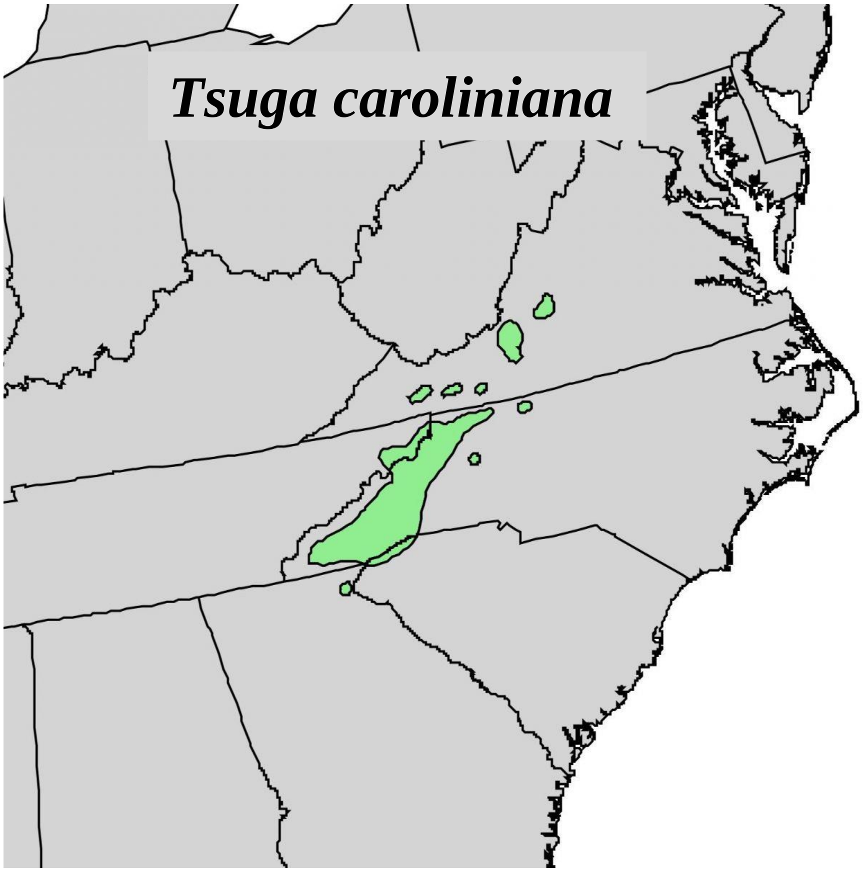


Figure 2. Native range of Carolina hemlock (USGS 2013).

coevolved with HWA. Thus they fare poorly when challenged with *A. tsugae* (McClure et al. 2001) because they are less resistant to HWA than many other hemlock species (Lagalante and Montgomery 2003), and native predator communities do not cause sufficient HWA population suppression (McClure 1987, Wallace and Hain 2000).

HWA are parthenogenic and have two generations each year: the sistens and progrediens generations (Figure 3). Sistens have a longer lifespan, living for approximately 9 mo. Sistens hatch in late spring and first instar nymphs (crawlers) settle on the base of needles, where they remain dormant for several months. They become active during the fall, progressing through several instars (Cheah et al. 2004). Mature sistens females produce 50-175 eggs each (McClure et al. 2001). Progrediens have a shorter, approximately 3 mo lifespan. Progrediens eggs, laid by the sistens generation, hatch in the early spring. Crawlers prefer to settle on the fresh, young hemlock tissue and that is reflected in higher survival and fecundity of HWA that feed on younger tissue (McClure 1991). Progrediens feed for a few months and lay eggs during late spring (Cheah et al. 2004). Each female progrediens can produce 25-125 eggs (McClure et al. 2001). Winged sexuparae are also produced alongside the progrediens. Sexuparae leave the hemlock in search of an alternate host, a spruce species not present in this country (McClure 1987, 1989). The proportion of sexuparae and progrediens produced is dependent on the HWA population density on a hemlock. As more sexuparae are produced, however, the HWA population experiences higher mortality, since they disperse and perish (Varty 1956, Eichhorn 1969).

In southern areas, HWA development occurs 1 mo sooner than in more northern areas (Grant et al. 2006), and HWA has been able to disperse more rapidly and cause more pronounced

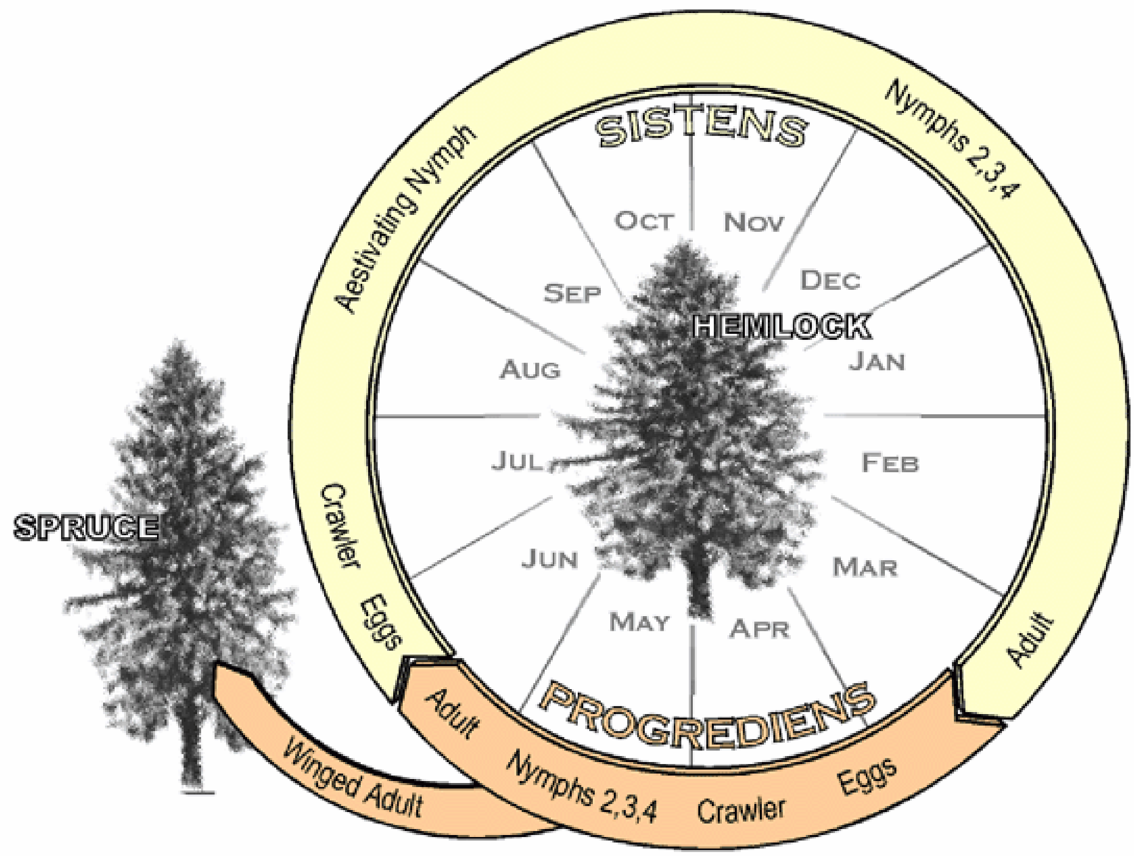


Figure 3. Hemlock woolly adelgid life cycle in North America (Cheah et al. 2004).

hemlock decline in the southern Appalachians (Skinner et al. 2003, Nuckolls et al. 2009). This phenomenon of accelerated HWA development and hemlock decline may be due to winter temperatures being insufficiently cold to kill large numbers of HWA (Skinner et al. 2003). Since little winter mortality occurs in this region, the movement of the HWA front is not restrained in the southern Appalachians (Ward et al. 2004).

Upon hatching, first instar nymphs locate the base of a hemlock needle from which to feed (Young et al. 1995). HWA have a piercing-sucking mouthpart, a stylet (McClure 1987), which they attach at the base of the hemlock needle, drawing fluid from the xylem ray parenchyma, which depletes critical storage tissues of the hemlock (Young et al. 1995). Since the carbohydrate and nutrients in the xylem ray parenchyma are depleted, less energy is available for tree growth, defense, metabolism, and reproduction (Shigo 1989). HWA alters the ability of eastern hemlock to photosynthesize (Nelson et al. 2014). Foliage infested with HWA exhibits decreased growth, reduced bud production, and lower water potential (Gonda-King et al. 2014). In addition, HWA infestations on hemlock foliage negatively alter the mechanical properties of needles by reducing mechanical strength and lowering twig flexibility (Soltis et al. 2014). HWA feeding also initiates a systemic hypersensitive response in hemlocks indicated by increased hydrogen peroxide production (Radville et al. 2011). Initial visible symptoms may include chlorosis and bud abortion (McClure 1991). Densities as low as four HWA feeding on 2 cm of twig can inhibit new foliage development (McClure 1991). Hemlocks exhibit little to no new foliage development, and as the infestation progresses, graying of the needles, branch mortality, and an increasingly thinner canopy occurs (McClure 1991, Orwig and Foster 1998, Jenkins et al. 1999, McClure and Cheah 1999, Stadler et al. 2005, Eschtruth et al. 2006).

HWA progresses through a multi-year population cycle during an infestation. Approximately 5 to 6 yr after initial infestation, the HWA population on a hemlock often crashes. However, once the hemlock begins to recover, HWA populations surge, most often resulting in hemlock mortality (Mayer et al. 2002). McClure (1991) documented a 4 yr cycle in which highest HWA densities occurred in the first and third year of an infestation. Density was dependent on patterns of new growth on the hemlock. In some cases, mortality occurs in as few as 2 to 3 yr after initial infestation, especially where winter temperatures do not limit HWA survival (Orwig et al. 2002, Nuckolls et al. 2009, Webster 2010). Eastern hemlocks are now infested with HWA throughout much of their native range, with the range continuing to expand northward and westward (Figure 4) (Lambdin et al. 2006, USDA Forest Service 2013), resulting in widespread hemlock mortality and decline. To date tens of millions of dollars have been invested in programs to suppress HWA and preserve hemlock resources (Aukema et al. 2011).

Hemlock Woolly Adelgid Management

Integrated pest management (IPM) is defined as "a decision support system for the selection and use of pest control tactics, singly or harmoniously coordinated into a management strategy, based on cost/benefit analyses that take into account the interests of and impacts on producers, society, and the environment" (Kogan 1998). IPM views the ecosystem as the management unit, rather than viewing the crop or resource of interest in isolation from its environment. The delicate balance of the environment, desired hemlock health, and the perception of society all factor into the decisions made to suppress HWA populations. IPM is not a system of eradication of pests but of lowering pest populations to acceptable levels below

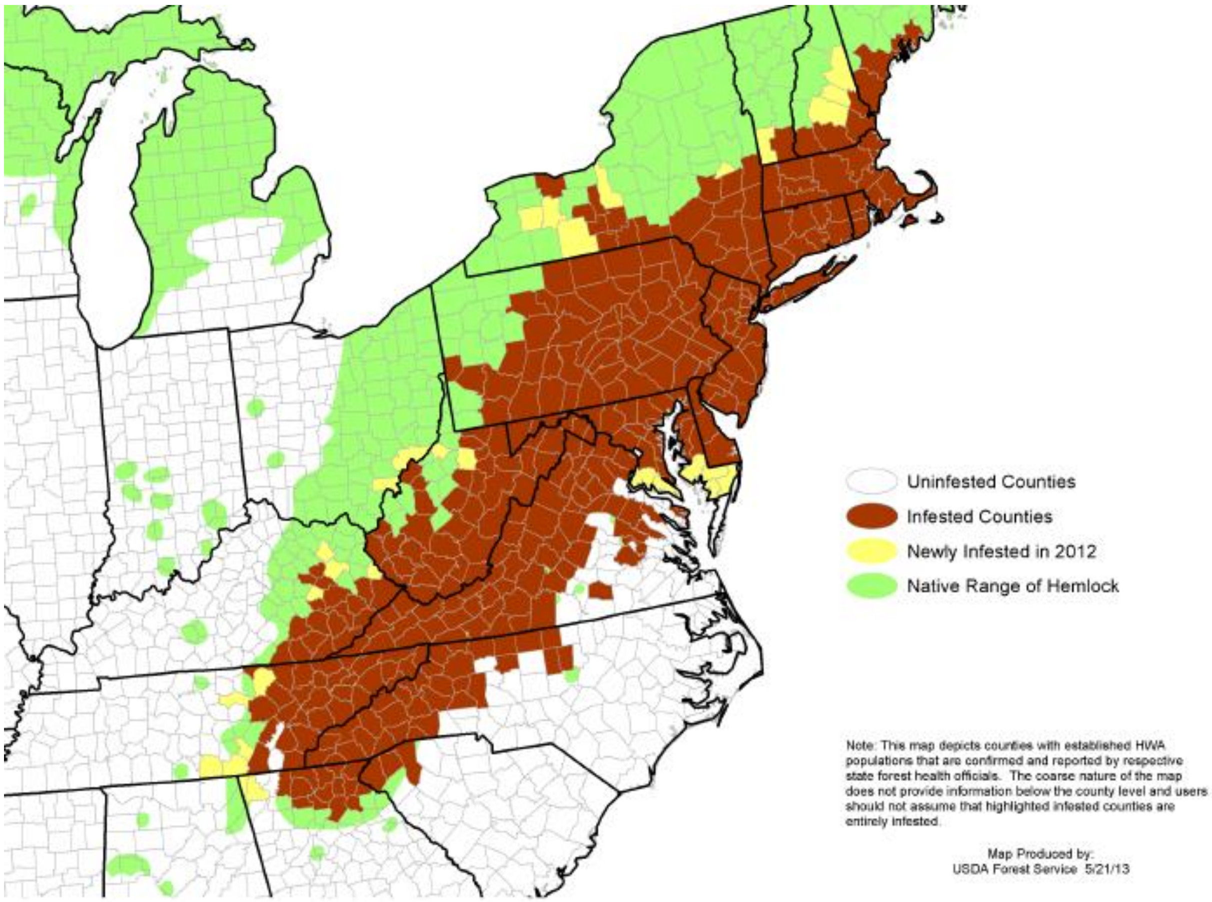


Figure 4. Distribution of hemlock woolly adelgid, eastern North America (USFS 2013).

economic thresholds, or in the case of hemlocks, thresholds to ensure continued hemlock health. Hemlock IPM programs utilize numerous control tactics to suppress HWA in eastern forests. A multi-faceted program may employ biological control and chemical insecticides, such as horticultural oil, insecticidal soap, and the neonicotinoid insecticides imidacloprid and dinotefuran (Webster 2010). Key to the IPM process is that chemical control of pests is a last resort. However, in hemlock systems insecticides are often the only viable option to combat HWA and provide immediate protection of hemlock resources.

Biological control projects have been initiated in numerous HWA management programs. Coleopteran biological control species from Asia and western North America have been released (Onken and Reardon 2011). Currently, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), a native to the northwestern United States and western Canada, and *Sasajiscymnus tsugae* (Sasaji and McClure) (Coleoptera: Coccinellidae), a native of Japan (Cheah et al. 2004), have been most commonly released. *L. nigrinus* has become established in locations at population levels sufficient to allow for collection for relocation of beetles to new release sites (Mausel et al. 2010, Onken and Reardon 2011). Other biological control species include *Laricobius osakensis* Montgomery (Coleoptera: Derodontidae), *Scymnus coniferarum* (Crotch) (Coleoptera: Coccinellidae), *Scymnnus sinuanodulus* Yu and Yao (Coleoptera: Coccinellidae), *Leucopis argenticollis* Zetterstedt (Diptera: Chamaemyiidae) and *L. piniperda* Malloch (Diptera: Chamaemyiidae) (Kohler et al. 2008, Darr and Salom 2014, Mooneyham and Salom 2014, USDA Forest Service 2015b). However, overall biological control has had limited success in establishment and effective HWA suppression in forest settings.

Imidacloprid and dinotefuran belong to a class of pesticides called neonicotinoids, which were originally developed for their improved toxicity profiles compared to older insecticide classes. Neonicotinoids are commonly used because they are selective for treating arthropod pests, have low fish and mammalian toxicity, and can be applied by various methods (Sánchez-Bayo and Hyne 2014). In 1991 imidacloprid was the first neonicotinoid introduced to the market (Nauen and Bretschneider 2002) and in 1994 was registered in the United States (USEPA 1994). Imidacloprid has been used in forestry settings for the suppression of many insect pests as early as 1994 (Steward and Horner 1994). In 2006 the patent for imidacloprid held by Bayer CropScience expired which allowed other companies to produce a generic version of the insecticide at a much lower cost. Imidacloprid treatments, which can be applied to hemlocks by numerous systemic soil application methods such as trunk injection, soil injection, soil drench, and CoreTect slow release pellets, have been highly effective in multi-year HWA suppression (Cowles and Cheah 2002, Cowles et al. 2006, Coots 2012, Coots et al. 2013, Eisenback et al. 2014, Mayfield et al. 2015). Due to costs of imidacloprid and the longevity of efficacy, imidacloprid is the most commonly used pesticide in HWA management programs.

Imidacloprid targets nerve synapses, functioning by irreversibly binding to acetylcholine receptors in post-synaptic nerve membranes in the insect central nervous system, causing the eventual termination of nerve impulses (Nauen and Bretschneider 2002). Imidacloprid can be effective through either ingestion or direct contact (Matsuda et al. 2001, Tomizawa and Casida 2005), which can result in insect mortality within 24–48 hr (Bai et al. 1991, Mullins and Christie 1995).

Once applied to the soil and absorbed by the plant, imidacloprid is metabolized into compounds with insecticidal properties, such as imidacloprid olefin, 5-hydroxy imidacloprid, and 4,5-di-hydroxy imidacloprid (henceforth referred to as olefin, 5-hydroxy, and dihydroxy, respectively) (Nauen et al. 1998) (Figure 5). Proposed metabolic pathways for imidacloprid indicate hydroxylation of imidacloprid to produce 5-hydroxy, which is then converted to olefin (Nauen et al. 1998, Sur and Stork 2003). The presence of these metabolites compounded with the effects of imidacloprid increases the degree of control for this product (Nauen et al. 1998). For example, olefin is about 15 times as toxic as imidacloprid to the green peach aphid, *Myzus periscae* (Sulzer) (Hemiptera: Aphididae), and the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) (Nauen et al. 1998). Olefin is over 14 times more toxic to HWA than imidacloprid (Coots 2012). Dose-response assays conducted in two separate studies have documented the LC_{50} (lethal concentration) of imidacloprid for HWA as 112 and 300 ppb (Cowles et al. 2006, Coots 2012), while the LC_{50} for olefin is 6 ppb (Coots 2012). The persistence and insecticidal properties of these compounds indicate a long-term effect of imidacloprid treatments, beyond the activity of the parent compound alone (Nauen et al. 1998, Cook 2008, Coots 2012).

The forest floor of areas with large hemlock populations has a thick organic surface layer where many of the absorptive roots of hemlocks are located (Cowles et al. 2006). Since imidacloprid binds to organic matter in the soil (Mullins and Christie 1995, Cox et al. 1998), the insecticide is kept in place and absorbed by the hemlock roots as it is immobilized (Cowles et al. 2006). Mass flow through the xylem is responsible for the absorption and transport of imidacloprid (Tattar et al. 1998, Castle et al. 2005, Byrne and Toscano 2006). The concentration

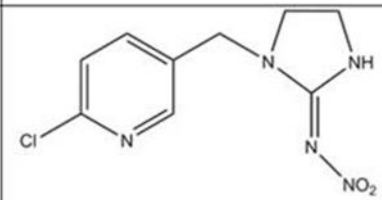
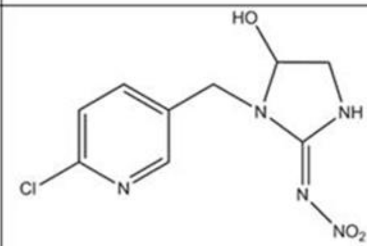
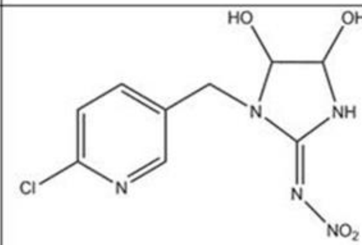
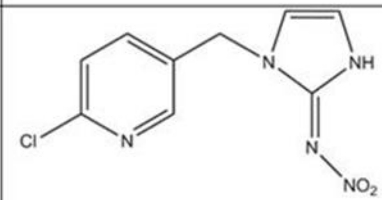
Common Name	IUPAC Name	Structure
Imidacloprid	1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine	
5-hydroxy	1-(6-chloro-3-pyridylmethyl)-2-(nitroimino)imidazolidin-5-ol	
Dihydroxy	1-(6-chloro-3-pyridylmethyl)-2-(nitroimino)imidazolidin-4,5-diol	
Olefin	1-(6-chloro-3-pyridylmethyl)-*N*-nitro-1,3-dihydro-imidazol-2-ylideneamine	

Figure 5. The IUPAC[1] names and chemical structures of imidacloprid and three insecticidal imidacloprid metabolites (5-hydroxy, dihydroxy, and olefin).

[1]IUPAC = International Union of Pure and Applied Chemistry

at which imidacloprid is maintained in the xylem is probably influenced by the amount of water transported through the xylem (Ford et al. 2007).

After soil applications, imidacloprid can translocate from the roots to the hemlock foliage in effective concentrations for HWA mortality in as little as 3 mo (Tattar et al. 1998, Coots et al. 2013). Imidacloprid concentrations within hemlock foliage peaks between 9 and 15 mo after soil application (Dilling et al. 2010, Coots et al. 2013). However, the concentrations of olefin continue to rise for as many as 3 yr after treatment providing continued HWA suppression (Coots et al. 2013). Presence of imidacloprid and its metabolites in hemlock over numerous years is likely dependent on initial imidacloprid uptake from the soil by hemlocks, since the highest concentrations of imidacloprid are present within 9 to 15 mo of application followed by reduced concentrations over time (Coots et al. 2013). Soil uptake may be affected by soil moisture conditions near the time of imidacloprid application, soil organic matter content, topography, and hemlock growth rate. Residual concentrations, which may be the result of imidacloprid and olefin, stored within hemlock tissue, continuing to move upwards in the sap over time, provide continuous HWA suppression over numerous years.

The objectives of HWA management programs determine which management tactics are employed and how they are implemented. Goals may include maximum suppression, maintenance of HWA populations below levels that cause reduced hemlock health, or integration of chemical and biological control tactics. Many recent studies have focused on obtaining a balance between chemical and biological options with the goal of maintaining HWA populations that are sufficient to support predator populations, but low enough to not negatively affect hemlock health (Eisenback et al. 2010, 2014, Joseph et al. 2011a,b, Mayfield et al. 2015).

However, while HWA management tactics applied to forest systems provide benefits to hemlocks, inherent risks to the surrounding ecosystem must be considered.

Non-Target Risks

Because of their high efficacy with insect pest populations, low toxicity to vertebrates, systemic activity, and ease of application, neonicotinoids have become the most commonly used insecticide class worldwide (Goulson 2013). The widespread use of neonicotinoids carries the risk of overuse on a global scale. Neonicotinoid use proliferates in nearly every facet of pest management including parasite treatment for pets, use in row cropping systems, prophylactic seed treatments, and pest prevention in forests. The common use of neonicotinoids has a great potential to stray far from the IPM tactics that have been developed since the post-World War II era overuse of pesticides (Goulson 2013).

New non-target risks of neonicotinoids have been discovered since imidacloprid was originally approved by the USEPA. Since imidacloprid has been on the market longer than other neonicotinoids, most research and public concern has focused on this compound. Factors of particular concern include environmental persistence, potential impacts to surface water quality, toxicity to aquatic macroinvertebrates and other non-target organisms, and potential role in pollinator decline (USEPA 2008b, Pestana et al. 2009, Cresswell 2011, Starner and Goh 2012, Goulson 2013).

Similar to other pesticides, once in the environment, imidacloprid begins to degrade by biotic, abiotic, and photolytic degradation (Wamhoff and Schneider 1999), and some of these degradation products of imidacloprid have insecticidal properties (Nauen et al. 1998, 1999). The

persistence and movement of imidacloprid and its metabolites in the environment will influence their potential to cause negative non-target impacts. Imidacloprid can be persistent in the soil as it has the capacity to easily bind to organic matter. The sorption of imidacloprid into soil is dependent on the concentration of imidacloprid and the organic matter content in the soil (Mullins and Christie 1995, Cox et al. 1998). In soils with high organic matter content, less imidacloprid movement through the soil column is expected (Cox et al. 1998). Highly variable half-life times of imidacloprid in soil have been shown, ranging from 28 to over 1,200 d (Baskaran et al. 1999, Sarkar et al.2001, Fernandez-Bayo et al. 2009). However, it is clear that due to its binding capacity and potentially long half-life in soil, imidacloprid has the ability to persist in soil for extended time periods. Accumulating levels of all neonicotinoids in soil due to repeated applications in successive years, as often happens in agricultural systems, is a concern (Goulson 2013).

According to the USEPA, imidacloprid movement through the soil is a route of potential impact to surface water quality (USEPA 2008b). While leaching is possible, limitations on the horizontal movement of imidacloprid in the soil when using soil injections exist, indicating that imidacloprid may be unable to move beyond the drip line of hemlock trees (Knoepp et al. 2010). Cowles (2009) recovered imidacloprid leaching from soil columns when conducting experiments where rainfall was simulated on soil columns dosed with imidacloprid. Other soil column studies have shown that imidacloprid has an ability to leach through soil (Perez et al. 1998, Pradas et al. 1999, Gupta et al. 2002).

In addition to leaching, imidacloprid and its metabolites may move into the water column through leaf degradation, since imidacloprid, olefin and 5-hydroxy have been detected in

hemlock foliage tissue (Dilling et al. 2010, Coots et al. 2013). A similar scenario has been documented in the laboratory using ash leaves, where imidacloprid was found to enter the water column as leaves from imidacloprid-treated ash trees degraded (Kreutzweiser et al. 2007). However, persistence of imidacloprid in water indicated that imidacloprid in the water column due to leaching has the potential to impact stream systems for longer time spans as compared to imidacloprid exposure from leaves falling from treated trees (Kreutzweiser et al. 2007).

Imidacloprid has been documented in surface water in numerous studies in both agricultural and forest settings (Churchel et al. 2011, Starner and Goh 2012, van Dijk et al. 2012). However, once imidacloprid does enter surface waters, its ability to persist may be limited, as it easily photodegrades (Moza et al. 1998). Documented half-life of imidacloprid in water ranges from as little as 1 h to 3 d (Agüera et al. 1998, Moza et al. 1998, Wamhoff and Schneider 1999, Redlich et al. 2007). Half-life can vary by season, ranging from estimates of 8.6 – 52.8 h (Lu et al. 2015). In the absence of light, imidacloprid is stable in water for more than 12 h. However, when exposed to light complete degradation has been documented in less than 5 h (Agüera et al. 1998).

Imidacloprid is toxic to aquatic macroinvertebrates. The concentration of imidacloprid necessary to cause mortality in aquatic organisms is much lower than that which is lethal to HWA. The LC_{50} for *Chironomus* species (Diptera: Chironomidae) has been reported from 5.40 - 19.90 ppb in 48 - 96 h dose-response laboratory exposure studies (Stoughton et al. 2008, Pestana et al 2009, Azevedo-Pereira et al. 2011a). Sublethal effects on *Chironomus* species were documented at concentrations of 0.50 to 3.00 ppb in 96 h exposure trials (Azevedo-Pereira et al. 2011b). Lethal concentrations for mayfly species (Ephemeroptera) have been documented

ranging from 0.65 – 8.49 ppb (Alexander et al. 2007, Beketov and Liess 2008). Unfortunately, laboratory experiments consisting of single species analyses are not adequate to fully gauge potential ecological threats (Crane 1997), thus the effects of imidacloprid on aquatic macroinvertebrate communities may be greater than anticipated.

In addition to concerns about imidacloprid contamination in soil and aquatic systems, the role all neonicotinoids may play in pollinator declines has received much scientific and public attention in recent years. Many other factors are involved with pollinator decline and colony collapse disorder, such as parasites, pathogens, and decreased resource diversity (Kaplan 2012). However, imidacloprid has been implicated in pollinator declines (Schmuck et al. 2001, Cresswell 2011). The LC_{50} for imidacloprid in honey bees is low at only 5 ng (oral) and 24 ng (contact) per bee. Mortality of honey bees at field-realistic concentrations has been documented (Suchail et al. 2000). Other colony effects such as reduced foraging efficiency, learning, colony growth, and queen production due to neonicotinoids have been observed (Yang et al. 2008, Gill et al. 2012, Henry et al. 2012, Whitehorn et al. 2012). However, as hemlocks are wind pollinated, risks to pollinators in hemlock systems are lower compared to other systems where imidacloprid is applied to insect-pollinated plants.

Possible non-target effects of imidacloprid specific to eastern hemlock systems in both terrestrial and aquatic habitats have been investigated by numerous researchers (Hakeem 2008, Dilling et al. 2009, Churchel et al. 2011). Imidacloprid applied to hemlocks by soil injection can move laterally and horizontally through the soil (Knoepp et al. 2012). In numerous studies imidacloprid has been documented in surface waters associated with soil applications of imidacloprid in agricultural areas (Starner and Goh 2012, Hladik et al. 2014, Main et al. 2014).

Although few studies have been conducted on potential impacts of imidacloprid on water quality in hemlock systems, imidacloprid has been documented, although in low concentrations, in hemlock systems (Churchel et al. 2011). One water sample collected 720 days post-treatment contained imidacloprid, and the authors concluded no negative effects to water quality and assessed aquatic macroinvertebrate communities from the imidacloprid treatments (Churchel et al. 2011).

HWA treatments with imidacloprid can have negative short-term effects on both canopy and soil-dwelling insects. Hakeem (2008) found fewer predators in the lower strata of hemlock canopies where imidacloprid concentrations were higher, when compared to the upper canopy, which had a lower concentration of imidacloprid and more predators. Soil drench treatments of imidacloprid caused greater reductions in non-target species richness and abundance of insects located within the hemlock canopy when compared to other imidacloprid application methods. The reductions in richness and abundance are primarily attributed to higher concentrations of imidacloprid in hemlock tissues (Dilling et al. 2007). Soil-inhabiting collembolans associated with eastern hemlock experienced a significant decrease in abundance and richness due to soil drench treatments of imidacloprid (Reynolds 2008).

The ultimate goal of HWA IPM programs is to provide the best possible HWA suppression while continuing to protect the entire forest system. As hemlocks are foundation species that contribute a wealth of ecosystem services to eastern forests, the preservation of the resource is imperative, however, the hemlock resource cannot be protected at the expense of the surrounding communities. Environmental risk is inherent in any pest management program, and those risks must be considered in developing management goals and objectives. Resource

managers with hemlock woolly adelgid suppression programs seek optimization of HWA management tactics while minimizing environmental risks of the selected tactics to protect the entire forest resource.

Great Smoky Mountains National Park HWA Management Program

In 2002, HWA was first documented in the Great Smoky Mountains National Park (GRSM) at ten sites; however, HWA was most likely present in the Park approximately 2 yr before it was documented (Lambdin and Grant 2003a, b). HWA is now present throughout GRSM wherever hemlocks are located. The range of eastern hemlock is extensive throughout GRSM with over 55,846 ha of hemlock. Over 600 ha are old growth hemlock stands, and more than 5,665 ha are forests that are dominated by hemlocks (Webster 2010). Given the cascading forest health effects of HWA damage and the magnitude of eastern hemlock presence in the Park, GRSM has the potential to suffer incalculable losses due to HWA induced mortality.

Once HWA was detected in GRSM, Park personnel launched an aggressive IPM program against HWA to control the invasive pest and preserve the hemlock resources. Biological control projects have been initiated in the Park using *S. tsugae*, and *L. nigrinus*. As of 2015 over 500,000 beetles were released in the Park (Jesse Webster, personal communication). Efforts to assess the effectiveness of releases are currently underway. Chemical treatments, using insecticidal soap, horticultural oil, dinotefuran, and imidacloprid, also have been implemented. Areas were prioritized for chemical treatment based on public access and ecological value. Commonly treated areas include roadways, trails, campgrounds, riparian areas, and conservation areas (Webster 2010). Imidacloprid was applied in GRSM as soil injections, basal drench, stem

injections, or as a dissolvable pellet (CoreTect). Over 250,000 trees, many in riparian areas, have received imidacloprid soil treatments (Jesse Webster, personal communication).

Since the initiation of their HWA management plan, Park personnel have maintained detailed records on imidacloprid treatments, including treatment dates, dosage rates, tree diameter at breast height (DBH) measurements, and location for individual hemlocks. The detailed records on past treatments have allowed the implementation of a retrospective research program to address knowledge gaps in HWA management.

Objectives

The objectives of this study are to investigate the effectiveness of imidacloprid treatments and to evaluate potential non-target stream impacts of the GRSM HWA management program. The first objective was to assess the concentration of imidacloprid and its insecticidal metabolites (dihydroxy, 5-hydroxy, and olefin) in hemlock foliage 4 - 7 yr post-treatment. The second objective was to assess the concentration of imidacloprid and its insecticidal metabolites in different DBH size classes of hemlocks 4 - 7 yr post-treatment and to develop a model to optimize imidacloprid doses based on hemlock diameter. The third objective was to examine HWA suppression among different DBH size hemlocks 4 - 7 yr after treatment and to assess associated hemlock canopy health. The fourth objective of this research project was to determine whether imidacloprid and its metabolites were present in streams associated with imidacloprid treatment areas and assess if treatment area and timing factors contribute to observed concentrations. The fifth objective was to assess aquatic macroinvertebrate community differences between sampling locations upstream and downstream from imidacloprid treatment

areas to determine if the use of imidacloprid is causing negative effects to aquatic communities. The overall goal of this research program was to provide research-based information to fill knowledge gaps to improve the GRSM HWA management program.

CHAPTER II. ASSESSMENT OF IMIDACLOPRID AND ITS METABOLITES IN FOLIAGE OF EASTERN HEMLOCK MULTIPLE YEARS FOLLOWING TREATMENT FOR HEMLOCK WOOLLY ADELGID, *ADELGES TSUGAE*, IN FORESTED CONDITIONS

This chapter is revised based on a paper published by Elizabeth Benton, Jerome Grant, Jesse Webster, Rebecca Nichols, Rich Cowles, Anthony Lagalante, and Carla Coots:

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My contributions to this paper include (1) reviewing pertinent literature, (2) designing and conducting experiments, (3) processing, analyzing, and interpreting data, and (4) the majority of the writing.

Abstract

Widespread decline and mortality of eastern hemlock, *Tsuga canadensis* (L.) Carrière, have been caused by hemlock woolly adelgid, *Adelges tsugae* (Annand) (HWA) (Hemiptera: Adelgidae). The current study is a retrospective analysis conducted in collaboration with Great Smoky Mountains National Park (GRSM) to determine longevity of imidacloprid and its insecticidal metabolites (imidacloprid olefin, 5-hydroxy, and dihydroxy) in GRSM's HWA integrated pest management (IPM) program. Foliage samples were collected from three canopy strata of hemlocks that were given imidacloprid basal drench treatments four to seven yr prior to sampling. Foliage was analyzed to assess concentrations in parts per billion (ppb) of imidacloprid and its metabolites. Imidacloprid and its olefin metabolite were present in most, 95% and 65% respectively, branchlets four to seven yr post-treatment, but the 5-hydroxy and dihydroxy metabolites were present in only 1.3% and 11.7% respectively, of the branchlets. Imidacloprid and olefin concentrations significantly decreased between four and seven yr post-treatment. Concentrations of both imidacloprid and olefin were below the LC_{50} for HWA five to

seven yr post-treatment. Knowledge of the longevity of imidacloprid treatments and its metabolite olefin can help maximize the use of imidacloprid in HWA IPM programs.

Introduction

Hemlock woolly adelgid, *Adelges tsugae* (Annand) (HWA) (Hemiptera: Adelgidae), a native of Japan (Havill et al. 2006), has caused widespread mortality in populations of eastern hemlock, *Tsuga canadensis* (L.) Carrière, and Carolina hemlock, *Tsuga caroliniana* Engelmann (Pinales: Pinaceae). HWA affects hemlocks by depleting carbohydrate storage (Young et al. 1995), reducing photosynthetic ability (Gonda-King et al. 2014, Nelson et al. 2014), reducing growth (Gonda-King et al. 2014), weakening twigs (Soltis et al. 2014), and initiating a hypersensitive response (Radville et al. 2011), eventually leading to tree death.

As a native species, eastern hemlock inhabits a distinctive niche and plays a vital ecological role in southern Appalachian forests as the only shade-tolerant conifer (Orwig and Foster 1998, Ward et al. 2004). Many animal species inhabit eastern hemlock and would be negatively impacted by its decline. For example, eastern hemlock systems have diverse arthropod communities, as more than 400 species of insects and numerous species of spiders are associated with this ecologically important native tree (Wallace and Hain 2000, Buck et al. 2005, Lynch et al. 2006, Dilling et al. 2007, 2009, Hakeem 2008, Mallis and Rieske 2011, Coots et al. 2012).

In the United States, HWA has two generations each year: the sistens generation and the progrediens generation. HWA adults are parthenogenic, and only females are produced by the sistens generation (Young et al. 1995). Sistens hatch in late spring and first-instar nymphs

(crawlers) settle on the base of needles, where they remain dormant for several months. They begin to feed and develop during the fall, progressing through several instars. In early spring sistens produce the progrediens generation eggs. Progrediens crawlers emerge and settle on the base of needles where they feed for several months. Progrediens produce eggs in late spring which hatch and begin the sistens generation again (Cheah et al. 2004).

Eastern hemlock has exhibited no widespread resistance against HWA (McClure 1995) and no native predators have sufficiently suppressed HWA populations (McClure 1987). Thus, eastern hemlock is now infested by HWA throughout most of its natural range in the eastern United States (Lambdin et al. 2006). Application of insecticides, particularly imidacloprid, has been essential in protecting and preserving eastern hemlock in the southern Appalachians. Imidacloprid, a neonicotinoid insecticide, functions by irreversibly binding to acetylcholine receptors in post-synaptic nerve membranes in the insect central nervous system, causing the eventual termination of nerve impulses (Nauen and Bretschneider 2002). Imidacloprid can be effective through either ingestion or direct contact (Matsuda et al. 2001, Tomizawa and Casida 2005), which can result in insect mortality within 24–48 hr (Bai et al. 1991, Mullins and Christie 1995). Once applied to the soil and absorbed by the plant, imidacloprid is metabolized into compounds with insecticidal properties, such as imidacloprid olefin, 5-hydroxy imidacloprid, and 4,5-di-hydroxy imidacloprid (henceforth referred to as olefin, 5-hydroxy, and dihydroxy, respectively) (Nauen et al. 1998). Proposed metabolic pathways for imidacloprid indicate hydroxylation of imidacloprid to produces 5-hydroxy, which is then converted to olefin (Nauen et al. 1998, Sur and Stork 2003). The presence of these metabolites, compounded with the effects of imidacloprid increases the degree of control for this product (Nauen et al. 1998). For

example, olefin is about 15 times as toxic as the parent compound to the green peach aphid, *Myzus periscae* (Sulzer) (Hemiptera: Aphididae), and the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) (Nauen et al. 1998). The persistence and insecticidal properties of these compounds indicate a long-term effect of imidacloprid treatments, beyond the activity of the parent compound alone (Nauen et al. 1998, Cook 2008, Coots 2012). Imidacloprid treatments have shown a high degree of success in reducing populations of HWA (Steward and Horner 1994, Doccola et al. 2003, Webb et al. 2003) for numerous years after application (Cowles et al. 2006, Cook 2008, Dilling et al. 2010, Coots 2012, Coots et al. 2013).

Soil applications of imidacloprid can provide residual insecticidal activity in hemlock trees for numerous years (Cowles et al. 2006, Coots 2012, Coots et al. 2013, Eisenback et al. 2014, Mayfield et al. 2015). This residual activity of imidacloprid in hemlock systems has been attributed to both the long retention of hemlock needles (approximately three yr) and the insecticidal capabilities of some of the metabolites of imidacloprid (Nauen et al. 1998, Schöning and Schmuck 2003). After soil applications, imidacloprid can translocate from the roots to the hemlock foliage in effective concentrations for HWA mortality in as little as three mo (Tattar et al. 1998, Coots et al. 2013). Imidacloprid concentrations within hemlock foliage peaks between nine and fifteen mo after soil application (Dilling et al. 2010, Coots et al. 2013). However, the concentrations of olefin continue to rise for as many as three yr after treatment providing continued HWA suppression (Coots et al. 2013).

The persistence of imidacloprid and olefin in hemlocks is a remarkable phenomenon. Since the highest concentrations of imidacloprid are present within nine to fifteen mo of application followed by reduced concentrations over time (Coots et al. 2013), it is likely that the

majority of imidacloprid is absorbed from the soil shortly after application. Residual concentrations may be the result of imidacloprid and olefin, stored within hemlock tissue, continuing to move upwards in the sap over time. Presence of imidacloprid and its metabolites in hemlock over numerous years is likely dependent on initial imidacloprid uptake by hemlocks (Coots et al. 2013), which may be affected by soil moisture conditions near the time of imidacloprid application, soil organic matter content, topography, and hemlock growth rate. HWA was first documented in the Great Smoky Mountains National Park (GRSM) in 2002 (Lambdin and Grant 2003a,b) and is currently present throughout GRSM wherever hemlocks are located. Eastern hemlock is distributed throughout GRSM, where more than 55,846 ha of hemlock are found. Of that, almost 607 ha are old growth hemlock and more than 5,665 ha are hemlock-dominated forests (Webster 2010). Changes in hemlock forests due to HWA damage include a diminished canopy (Orwig et al. 2008), greater light infiltration to the forest floor (Eschtruth et al. 2006), a drier forest floor (Orwig et al. 2008), altered nitrogen cycling in the soil (Jenkins et al. 1999), and more downed woody debris due to hemlock mortality (Orwig and Foster 1998). GRSM has the potential to suffer the loss of eastern hemlocks and the ecological functions they provide throughout the park due to HWA-induced mortality.

Once HWA was detected in GRSM, Park personnel began a concerted effort using various chemical treatments (such as insecticidal soap, horticultural oil, imidacloprid, and dinotefuran) and biological control to manage this invasive pest and preserve the hemlock resources. Biological control projects focused primarily on releases of *Sasajiscymnus tsugae* (Sasaji and McClure) (Coleoptera: Coccinellidae) and *Laricobius nigrinus* (Fender) (Coleoptera: Derodontidae). Numerous biological control beetle releases have been made in GRSM as part of

their integrated pest management program (Webster 2010, Hakeem et al. 2010, 2013). Areas for chemical treatment were prioritized based on public access and ecological value. Commonly treated areas included roadways, trails, campgrounds, riparian areas, and conservation areas (Webster 2010). Imidacloprid has been a major component of the HWA management plan in the Park, as more than 225,000 individual trees in GRSM have been treated with imidacloprid.

Since the initiation of their HWA management plan, Park personnel have maintained detailed records on treatments, including treatment dates, dosage rates, and tree measurements. Questions have arisen about how long treatments will be effective, how often treatments should be made, what are the optimal doses for different size hemlocks, is imidacloprid translocated evenly throughout the canopy, and do treatments provide even control throughout the canopy? To address these questions, a multi-year retrospective study was designed to provide information to enhance management of HWA. The objectives of this part of the study were to evaluate the presence, longevity, distribution within the canopy (lower, middle and upper strata), and temporal shift in composition of imidacloprid and its metabolites, olefin, 5-hydroxy, and dihydroxy, in hemlocks four to seven yr after a single imidacloprid treatment. Further analyses for future publications from this retrospective study will explore the concentrations of imidacloprid and its metabolites in hemlocks in differing diameter size classes, as well as population suppression of HWA and hemlock health numerous years after imidacloprid treatment.

Materials and Methods

Site/Tree Selection. Previous studies have focused on hemlock trees of similar sizes. However, this retrospective analysis aims to assess the longevity of imidacloprid treatments from a range of hemlock sizes found in a typical forest management program to better inform comprehensive management decisions. Potential study sites were selected based upon information in the GRSM hemlock treatment database, discussions with Park personnel, and site visits. To isolate the effects of single imidacloprid treatments, sites where hemlocks were treated once with imidacloprid were of interest. Due to sites receiving multiple treatments or treatments with additional pesticides, site availability in GRSM was limited. Final site selections were based on treatment method, time of treatment, geographic location, elevation, and hemlock forest composition (hemlock dominant or co-dominant). Sites where hemlocks received imidacloprid treatments four to seven yr before this project was initiated were selected. To increase the likelihood that sites experienced similar environmental conditions, close geographic proximity and similar elevations were also important factors in site selection. The elevation (10-m resolution) of each hemlock tree was determined using ArcMap 10 (Environmental Systems Resource Institute [ESRI] 2010). Because a drought occurred during the summer of 2007, and to ensure that all study trees experienced similar stressors, sites that had been treated with imidacloprid prior to the drought were chosen for this study.

Eastern hemlock trees (*n*=102) were selected at two sites (Anthony Creek [35° 35.682 N, -83° 45.845 W] and Hesse Creek [35° 40.190 N, -83° 52.126 W]) on the western side of GRSM and one site on private land (Mountain Homes, Inc. [35° 40.574 N, -83° 52.144 W]) adjacent to the Park (Table 1, Figure 6). Hemlocks at each site were sampled in 2012 and 2013.

Table 1. Characteristics of imidacloprid treatment sites, number of trees sampled at each site, treatment dates, and sampling dates, Great Smoky Mountains National Park, 2012.

Site	Elevation (m)[1]	Slope (degrees)	Aspect (degrees)	No. Trees Sampled per Site	Treatment Date[2]	Sampling Date[3]	
						2012	2013
Anthony Creek	671	18-22	350-50	34	Aug. – Oct. 2006	11, 18 Jan.	23, 24 Jan.
Hesse Creek	311	20	230-280	35	Dec. 2006	25 Jan., 1 Feb.	7 Feb.
Mountain Homes, Inc.	350	40-50	330-15	33	Dec. 2007	19, 24 Jan.	28, 29 Jan.

[1]Average elevation in meters at each site.
[2]Date imidacloprid was applied to soil underneath hemlock trees.
[3]Date branchlet samples were collected for analysis.

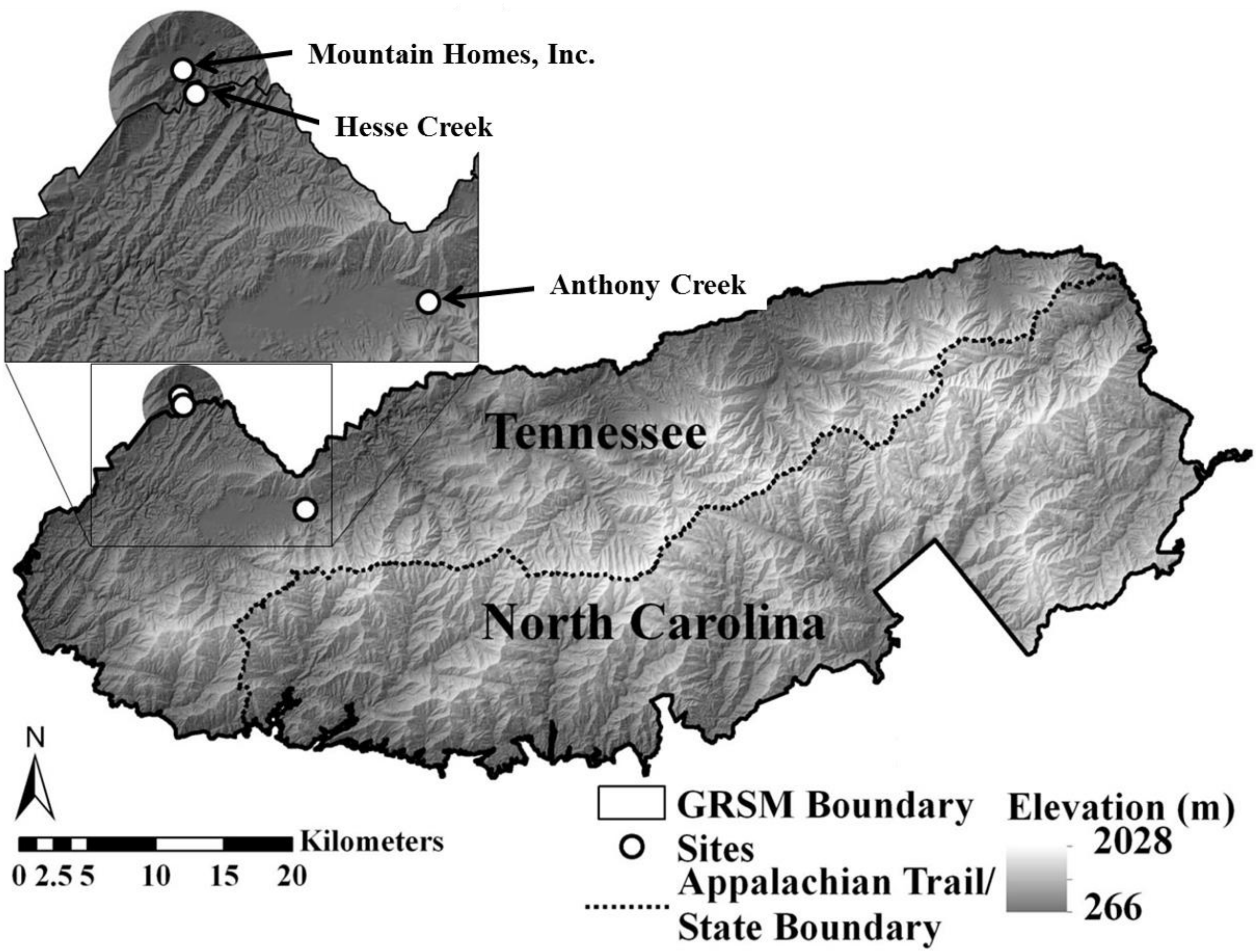

Figure 6. Location of sampling sites in the Great Smoky Mountains National Park (GRSM).

Imidacloprid treatments were applied at Mountain Homes (*n* = 33 trees, 17 ha, elevation: 350 m) in December 2007, four yr before initiation of sampling in January 2012 (Table 1). Treatments at Hesse Creek (*n* = 35 trees, 6 ha, elevation: 311 m) were applied in 2006, five yr before sampling began. Hemlocks at Hesse Creek and Mountain Homes, Inc. would have had effective concentrations of imidacloprid in the spring following treatment. Trees at Anthony Creek (*n* = 34 trees, 21 ha, elevation: 671 m) received imidacloprid treatments in 2006, five and one-half yr before sampling began in 2012 (Table 1). Treatments were applied to eastern hemlock at Anthony Creek during the late summer and early fall of 2006. Since imidacloprid can reach effective concentrations in the canopy at three mo post-treatment (Tattar et al. 1998, Coots et al. 2013), the Anthony Creek site will be classified as six yr post-treatment in 2012 and seven yr post-treatment in 2013 throughout the remainder of this paper. At each site, hemlocks ranged from 30.5 to 76.2 cm diameter at breast height (DBH) and ranged from 18.3 to 36.6 m tall. The DBH of hemlocks were evenly distributed among sites. Trees were marked with metal identification tags, as well as flagging tape, to enhance identification of study trees. As the yr post-treatment ranged from 4 to 6 yr in 2012 and 5 to 7 yr in 2013, more trees were sampled 5 and 6 yr post-treatment as compared to 4 and 7 yr post-treatment because of the overlap in yr post-treatment between 2012 and 2013.

Insecticide Application. Hemlocks were treated with a basal drench of imidacloprid (material is poured into the soil within 0.6 m of the base of the hemlock trunk) during 2006 and 2007 (Table 1). Imidacloprid dosage varied among sites and hemlock sizes. All hemlocks at Anthony Creek received a low dose imidacloprid treatment (0.7 g active ingredient [AI]/2.5 cm DBH). At Hesse Creek and Mountain Homes, hemlocks less than 63.5 cm DBH were given the same low dose

imidacloprid treatment (0.7 g AI/2.5 cm DBH), while trees 63.5 cm DBH and greater were given a high dose treatment (1.4 g AI/2.5 cm DBH). This dosage increase on larger trees at Hesse Creek and Mountain Homes was part of the HWA management program at both locations (Jesse Webster, personal communication).

Foliage Sampling and Canopy Stratification. Three branchlet samples (0.5 m long) were randomly collected from each of three strata (lower third, middle third, and upper third) of the live canopy of each hemlock selected at each site during the winters of 2012 and 2013 (Table 1) to assess the translocation of imidacloprid and its insecticidal metabolites, olefin, 5-hydroxy, and dihydroxy, within the canopy. The amount of live canopy varied widely among trees, depending upon height, dominance/codominance, etc. For example, heights from the ground surface to the bottom of the live canopy ranged from approximately 1.5 to 30.0 m, with some of the large trees (i.e., 30.0 m) having a small live crown ratio (i.e., 30%). Due to the height of the hemlocks and the remote location of the sites, tree climbers (Appalachian Arborists and GRSM personnel) ascended the trees with pre-labeled plastic bags (7.5 liter) and collected foliage samples using pole pruners. However, some lower stratum branchlet samples were collected with hand pruners, where possible. Branchlets were randomly selected for collection in each stratum of the tree. As samples were collected, they were placed into plastic bags and lowered to the ground in cloth buckets (approximately 19 liters). All samples from each tree were placed in plastic bags (49 liter) for transport to the laboratory. Once in the laboratory samples were placed in a walk-in cooler (4°C) for as many as 5 d to prevent mold growth on the foliage until sample preparation.

Sample Preparation. Branchlets were removed from the cooler and clipped into smaller sections (approximately 25 cm) within 5 d of arrival to the laboratory, and branchlet samples

were placed in labeled brown paper bags (2,900 cm^3) for drying. Samples were air dried at 21°C until needles easily detached from the twigs (approximately two wk). Dried needle tissue was pulverized using a Mr. Coffee™ coffee grinder (IDS77-NP, Rye, NY) within three mo of needle detachment. Processed samples were then placed in centrifuge tubes (50 mL) and stored in a dark, dry location. Pulverized needles (approximately 1 g) were placed in centrifuge tubes (15 mL) (Fischer Scientific, USA) and 10 mL of acetonitrile was added. A 1:10 (needle:solvent) ratio was used to extract the compounds from the hemlock needles as this ratio is adequate for needle extraction (Cowles et al. 2006). The centrifuge tube was shaken overnight on an orbital bench shaker (New Brunswick Scientific, Edison, NJ; model G33). Approximately 200 µL of supernatant acetonitrile was then passed directly through a 0.2 µm nylon filter into an autosampler vial containing a 300 µL vial insert for liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis. Spike recovery experiments provided sufficient recoveries (91-102%) for the target compounds of interest (Cook 2008).

Source of Chemicals. Imidacloprid was purchased from Supelco and the imidacloprid metabolites (olefin, 5-hydroxy, and dihydroxy) were provided by Bayer Agrochemical. These chemicals were used as standards in chemical analysis of hemlock foliar samples. Acetonitrile was HPLC grade (Fisher Scientific). All chemicals were used as received.

LC/MS/MS. Imidacloprid and its metabolites were quantified at Villanova University by LC/MS/MS. A Shimadzu Prominence HPLC system consisting of binary Shimadzu LC-20AD pumps and SIL-20A autosampler (Shimadzu, Colombia, MD) was used under Analyst software control (Applied BioSystems/SCIEX, Framingham, MA) for HPLC separation. A Phenomenex Gemini NX (C_{18}, 4.6 × 250 mm, 5 µm particle) fitted with a 2 mm guard column was used for

separation. An aqueous (water with 10 mM ammonium formate) phase and an organic (acetonitrile) mobile phase were used at a total flow of 1.0 mL min^{-1}. A gradient programmed elution was used that ramped from 25 to 95% acetonitrile (1-8 min), used a column wash with 95% acetonitrile (8-9.5 min), and had a column stabilization period with 25% methanol prior to the next injection. A 10 µL injection volume was used for imidacloprid and metabolite standards and the samples that were analyzed by LC/MS/MS.

Mass spectrometry was performed with an Applied BioSystems/SCIEX 3200 Q-TRAP triple quadrupole mass spectrometer (Framingham, MA) operated in positive electrospray ionization (ESI) mode. Multiple reaction monitoring (MRM) transitions were optimized using standards (Table 2) (Cook 2008). Optimized ESI source parameters were as follows: curtain gas (CUR) = 35 psi, CAD gas = medium, ESI nebulizer gas (GAS1) = 60 psi, auxiliary gas (GAS2) = 60 psi, ESI probe temperature = 550 °C, and ion spray voltage (IS) = 5500 V. The collision cell exit potential (CXP) was maintained at 4 V and the Q0 entrance potential (EP) was maintained at 10 V for all compounds. The resolution of Q1 and Q3 was fixed at high resolution and the dwell time for each MRM transition was 500 ms.

Analytical MS precursor and fragment ions and their optimized voltages as well as the analytical sensitivity of the LC/MS/MS method are summarized in Table 2. Standards for each compound were analyzed in the ranges provided. For each compound the concentration (ppb) limit of detection (LOD) is calculated at a signal-to-noise ratio of 3 based upon a low-concentration standard (Table 2). This concentration LOD is converted to an on-column mass LOD using the injected volume (10 µL for all compounds).

Table 2. Optimized precursor and fragment ions and voltages for imidacloprid and the metabolites of interest and analytical sensitivity. Standard errors were calculated at the 95% confidence interval of the mean.

Compound	Precursor Ion (*m/z*)	Fragment Ion (*m/z*)	Declustering Potential (V)	Collision Energy (V)	Calibration standard range (ppb)	LOD (ppb)	On-column LOD (pg)
Imidacloprid	256.1	209.1	27	26	336-12.4	0.4±0.1	4.5±1.2
Olefin	254.1	205.1	26	27	321-11.9	1.0±0.3	10.5±3.2
5-Hydroxy	272.2	191.1	32	22	255-9.5	1.3±0.6	13.1±5.7
Dihydroxy	288.1	207.2	35	21	544-60.5	31.2±7.8	312±78

Data Analysis. All data were entered in an Excel file (Microsoft, Redmond, WA). Data points below the LOD for each chemical were given a zero value. The LOD for olefin is 1.05±0.32 ppb, and the LC_{50} of olefin for HWA (6 ppb), determined in laboratory study through 15 d exposure duration (Coots 2012). Using the LOD rather than zero could artificially inflate averages relative to this LC_{50}. Because management recommendations will be made from the analysis of these data, the most conservative approach was used. Chemical concentration data were tested for normality using Shapiro-Wilks and Kolmogorov-Smirnov tests. Due to the wide range of imidacloprid and olefin concentrations, log transformations (ln[x + 0.1]) were used on these data prior to analysis. Boxplots were generated for both imidacloprid and olefin concentrations for years post-treatment to illustrate the wide range of data distribution. Mixed model ANOVA and least significant difference (LSD) mean separation procedure were conducted on imidacloprid and olefin concentration data ($P < 0.05$) using the GLIMMIX procedure in SAS (SAS Institute 2008). This analysis was conducted to determine differences between sampling years (2012 and 2013) and among sampling sites (Anthony Creek, Hesse Creek, and Mountain Homes) with tree × sampling year × site as a random effect. A separate analysis was conducted to determine differences among years post-treatment (four, five, six, and seven) and canopy strata (lower, middle, upper) with sampling year and sampling year × tree × years post-treatment as random effects. All means presented in results are back-transformed from the means of log-transformed data used in analyses.

Results and Discussion

Presence and Distribution of Imidacloprid and Its Metabolites. Imidacloprid and its metabolites were recovered from hemlock foliage for as many as seven yr after treatment. The majority of branchlets sampled during 2012 and 2013 contained imidacloprid (95.0%) and olefin (65.5%) concentrations above their LOD. However, few branchlets had detectable concentrations of 5-hydroxy (1.3%) and dihydroxy (11.7%). Thus, the 5-hydroxy and dihydroxy metabolites four to seven yr post-treatment are unlikely contributors to HWA suppression, and were not included in data analyses. The remainder of this paper focuses on imidacloprid and olefin as these chemicals are present in most of the branchlets sampled.

A wide range of imidacloprid (Figure 7) and olefin (Figure 8) concentrations (0 to 2,371 ppb and 0 to 1,134 ppb, respectively) was observed. Concentrations were more widely distributed for both imidacloprid and olefin in the four yr post-treatment group compared to the five, six, and seven yr post-treatment groups. Some branchlets had high concentrations of imidacloprid and olefin in excess of 500 ppb. Similarly high concentrations of imidacloprid have been observed by Cook (2008) and Eisenback et al. (2010). Forty branchlets had concentrations of imidacloprid exceeding 500 ppb, and 82% occurred in trees four yr post-treatment. In addition, 10 of the 13 branchlets with olefin concentrations in excess of 500 ppb were from samples collected four yr post-treatment. The branchlets with high olefin concentrations generally had high imidacloprid concentrations. Twelve of the 13 branches with olefin concentrations above 500 ppb, concentrations of imidacloprid in excess of 500 ppb, while all branchlets with imidacloprid concentrations in excess of 500 ppb, contained olefin concentrations greater than 150 ppb.

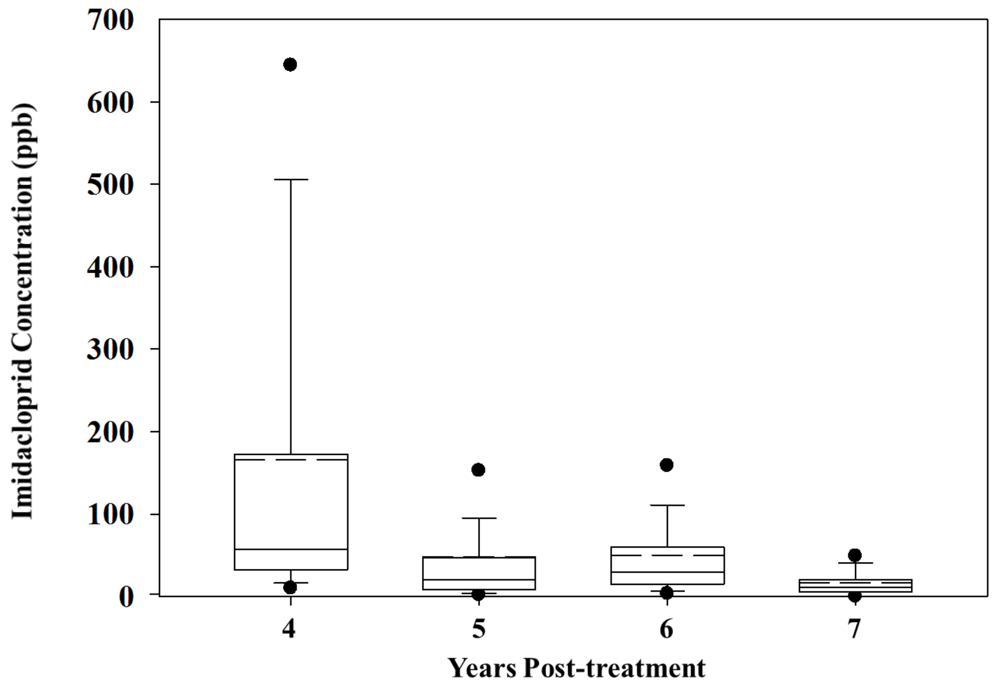

Figure 7. Distribution of imidacloprid concentration data four to seven years after basal drench treatments.

Boxes contain the 25th to the 75th percentiles of the data. Endpoints of the whiskers indicate the 10th and 90th percentiles of the data. Circles indicate the 5th and 95th percentiles of the data. The median is indicated by a solid line in each box. The dashed line indicates the arithmetic mean.

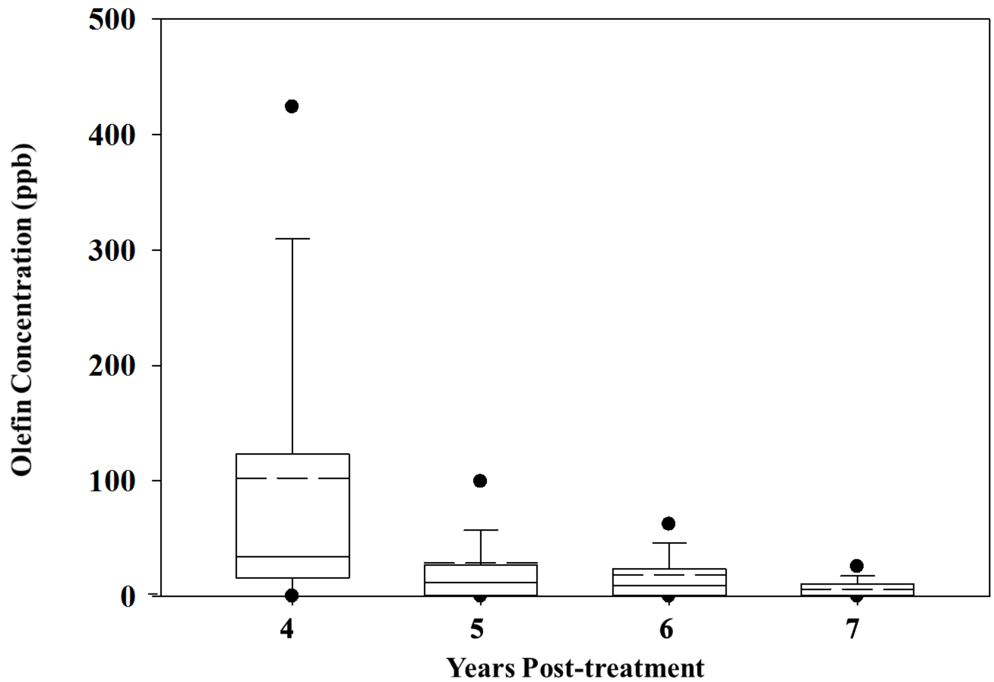

Figure 8. Distribution of olefin concentration data four to seven years after basal drench treatments.

Boxes contain the 25th to the 75th percentiles of the data. Endpoints of the whiskers indicate the 10th and 90th percentiles of the data. Circles indicate the 5th and 95th percentiles of the data. The median is indicated by a solid line in each box. The dashed line indicates the arithmetic mean.

Imidacloprid Concentration – Site and Sampling Year. Imidacloprid concentrations in branchlets were significantly different between 2012 and 2013 (F = 121.99; df = 1, 199; P < 0.001) (data not shown). Concentrations of imidacloprid were significantly higher in 2012 compared to those in 2013 (P < 0.05, LSD test). There were site-specific differences in concentration of imidacloprid (data for sampling years combined) (F = 8.78; df = 2, 199; P < 0.001) as well as site × sampling year interaction for imidacloprid concentrations (F = 8.99; df = 2, 199; P < 0.001). While each site had a higher concentration of imidacloprid in 2012 compared to 2013, the concentration of imidacloprid in foliage collected from hemlock at Mountain Homes was significantly higher than imidacloprid concentrations at all other sites in 2012 but significantly lower than Hesse Creek in 2013 (P < 0.05, LSD test) (Figure 9A). Imidacloprid concentrations at Anthony Creek in 2013 were not significantly different than those at Mountain Homes, but were significantly lower than those at Hesse Creek (P < 0.05, LSD test). The significant reduction in imidacloprid concentrations in foliage at Mountain Homes from 2012 to 2013 may be influenced, in part, due to a small number of branchlets with high concentrations of imidacloprid in 2012.

Dose-response assays have documented the LC_{50} of imidacloprid for HWA as 112 and 300 ppb (Cowles et al. 2006, Coots 2012), from laboratory experiments that by necessity involved exposure of 15 d and 20 d, respectively. In forest settings suppression of the HWA progrediens generation has been observed when imidacloprid concentrations are greater than 120 ppb two yr post-treatment (Cowles et al. 2006). In the current study, the highest average imidacloprid concentration per site and year post-treatment documented was 69.4 ppb from Mountain Homes in 2012, four yr post-treatment. The average imidacloprid concentrations at all

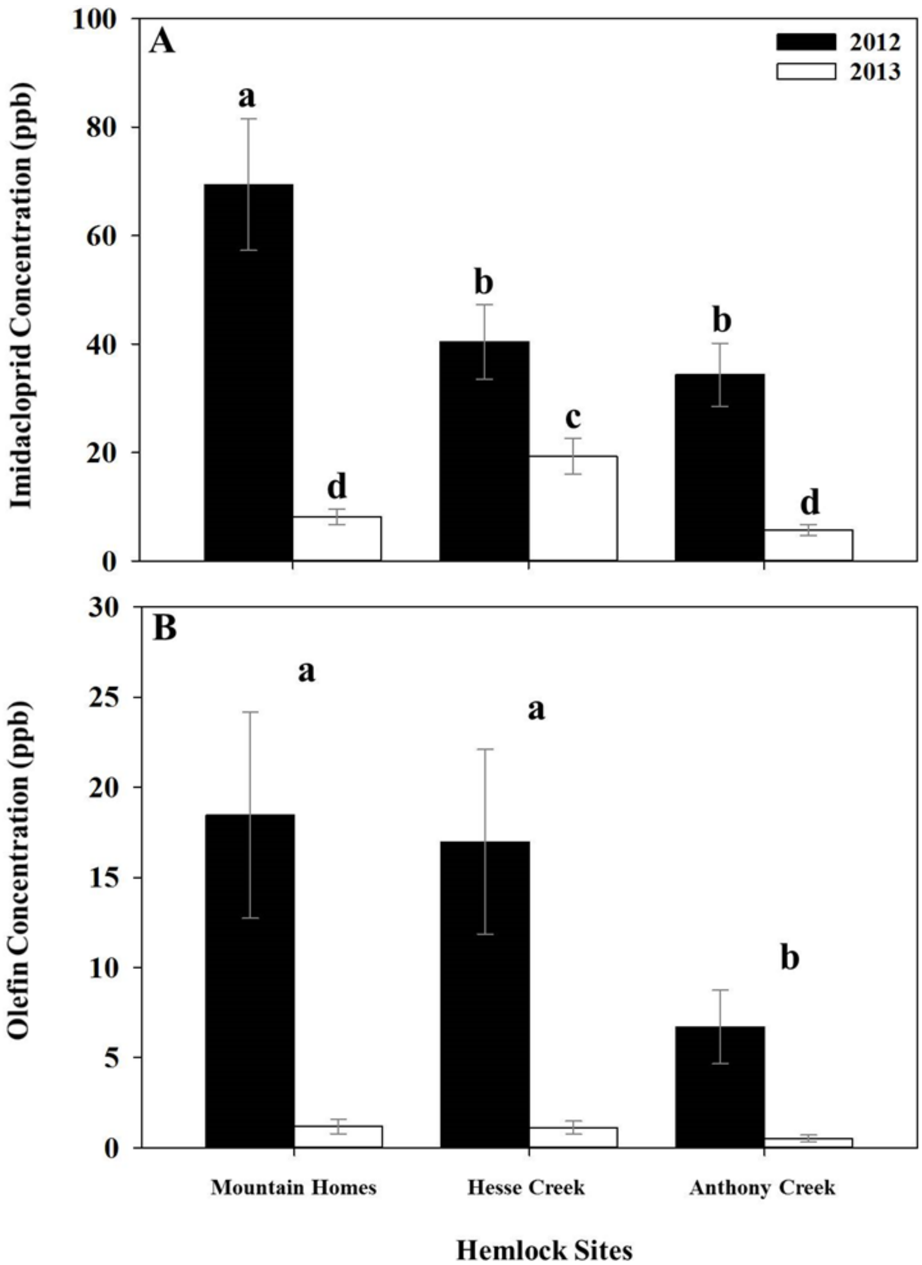

Figure 9. Comparison of imidacloprid (A) and olefin (B) concentrations in hemlock foliage in 2012 and 2013.

Means displayed are back-transformed from log transformed means used in the statistical analysis. Bars on each column denote standard error of the mean. Columns with the same letters are not significantly different ($P < 0.05$, LSD test). An interaction effect exists between the sites and the year of sampling for imidacloprid concentrations (A). Olefin concentrations had no interaction effect among the sites and between the year of sampling (B).

sites during 2012 and 2013 were below the 15 and 20 d LC_{50} for HWA, so while the imidacloprid that is present may contribute to HWA population suppression, the concentration of imidacloprid in foliage tissue appears insufficient to cause HWA mortality.

Olefin concentration – Site and Sampling Year. Overall average olefin concentration in 2012 (12.8 ± 2.3 ppb) was significantly different from the average olefin concentration in 2013 (0.88 ± 0.17 ppb) (F = 108.24; df = 1, 199; P < 0.001). Concentrations in 2012 were 14× higher than concentrations in 2013. Olefin concentrations in foliage at each site were higher in 2012 compared to 2013 (P < 0.05, LSD test) (Figure 9B). Olefin concentrations differed among sites (F = 5.13; df = 2, 199; P = 0.007) (Figure 9B), and no significant interaction between site and sampling year was detected (F = 0.11; df = 2, 199; P = 0.895). Since an interaction effect was not detected, differences among sites are based on olefin concentrations from 2012 and 2013 combined. Hesse Creek and Mountain Homes, the more recently treated sites, had higher concentrations of olefin than Anthony Creek (P < 0.05, LSD test).

More branchlets with high olefin concentrations and detectable concentrations of olefin were present in 2012 compared to 2013. While some branchlets sampled in 2013 had higher olefin concentrations, that occurrence was not observed to the degree seen in 2012. In addition, reductions in the percentage of branchlets with concentrations above the LOD for olefin were observed between 2012 and 2013. In 2012 83.5% of branchlets contained olefin concentrations above the LOD, while only 46.4% did in 2013. The observed reduction of branchlets with high olefin concentrations and a reduction in the number of branchlets with olefin at detectable concentrations explain the lower concentration found in 2013.

Olefin concentrations from all sites in 2012 exceeded the 15 d LC_{50} for HWA. The 15 d LC_{50} and LC_{80} of olefin for HWA are 6 and 7 ppb, respectively (Coots 2012). Olefin concentrations at all sites in 2012 were above 6 ppb, so sufficient levels of olefin were present at all sites in 2012 to contribute to moderate to high levels of HWA mortality. Hemlocks from Hesse Creek and Mountain Homes experienced olefin concentrations above the 15 d LC_{80} in 2012. However, the average olefin concentration from samples in 2013 was lower than those in 2012. The highest average olefin concentration observed in 2013 was only 1.2 ppb from Mountain Homes. The concentration of olefin at each site in 2013 was below the 15 d LC_{50} for HWA.

Imidacloprid Concentration – Years Post-treatment and Canopy Strata. Significant differences in average imidacloprid concentrations among years post-treatment were detected ($F = 8.60$; df = 3, 201; $P < 0.001$). Imidacloprid concentrations four yr post-treatment were significantly higher than imidacloprid concentrations five, six and seven yr post-treatment ($P < 0.05$, LSD test) (Table 3). High concentrations in relatively few branchlets collected four yr post-treatment may have influenced these results. Concentrations of imidacloprid seven yr post-treatment were significantly lower than average imidacloprid concentrations four, five, and six yr post-treatment ($P < 0.05$, LSD test). No significant difference between imidacloprid concentrations five and six yr post-treatment was detected ($P > 0.05$, LSD test). The concentrations of imidacloprid in foliage decreased from four to seven yr post-treatment, so that as time since treatment increases the concentration of imidacloprid decreases. Similar observations of decreases in imidacloprid concentrations at sites over time have been observed by Coots et al. (2013) where imidacloprid peaked at approximately one yr after treatment, then

Table 3. Comparison of imidacloprid and olefin concentrations in foliage four to seven years after imidacloprid treatment.

Years Post-Treatment	*N*	Imidacloprid Concentration (ppb)	Olefin Concentration (ppb)
4	33	41.05 ± 23.33a	6.36 ± 7.23a
5	68	18.31 ± 10.03b	4.61 ± 5.12ab
6	69	25.75 ± 14.08b	2.76 ± 3.11bc
7	34	9.74 ± 5.57c	1.65 ± 1.96c

Means (±SE) within a column followed by the same letters are not significantly different ($P < 0.05$, LSD test). Means displayed are back-transformed from log transformed means used in the statistical analysis. Significance is determined by standard error of the difference between means.

experienced a steady decline until three yr post-treatment when the study ended. In addition, imidacloprid concentrations are below the 15 and 20 d LC_{50} for HWA four to seven yr post-treatment.

Significant differences in imidacloprid concentrations among hemlock canopy strata, data combined for all years post-treatment ($F = 9.02$; df = 2, 1632; $P < 0.001$), were detected (Table 4). The middle stratum of the hemlock canopy exhibited the highest concentration of imidacloprid ($P < 0.05$, LSD test). Concentrations of imidacloprid were not significantly different in the lower and upper strata ($P > 0.05$, LSD test). No significant interaction effect between years post-treatment and canopy strata was observed (Table 5). While this study found higher concentrations of imidacloprid in the middle stratum of the hemlock canopy, Coots et al. (2013) observed higher concentrations of imidacloprid in the lower stratum of hemlocks. The differences in levels of imidacloprid among canopy strata could be due to site specific parameters or the size of hemlocks sampled in the studies. The hemlocks sampled in Coots et al. (2013) were 23.5 to 29.5 cm DBH, while hemlocks sampled in this study were 30.5 to 76.2 cm DBH. Thus, tree size may be a factor in the translocation of imidacloprid throughout the canopy.

Olefin Concentrations – Years Post-Treatment and Canopy Strata. Olefin concentration decreased as years post-treatment increased ($F = 2.91$; df = 3, 201; $P = 0.036$) (Table 3), and no significant difference in olefin concentrations among canopy strata was detected ($F = 1.53$; df = 2, 1632; $P = 0.218$) (Table 4). However, significant interactions between years post-treatment and canopy strata for olefin concentrations were detected ($F = 2.39$; df = 6, 1632; $P = 0.026$). Olefin concentrations among canopy strata or years post-treatment in samples collected four and five yr post-treatment were not significantly different ($P > 0.05$, LSD test) (Table 5). Olefin

Table 4. Comparison of imidacloprid and olefin concentrations in foliage in each canopy stratum four to seven years after imidacloprid treatment.

Canopy Stratum	Imidacloprid Concentration (ppb)	Olefin Concentration (ppb)
Lower	20.02 ± 10.81b	3.37 ± 3.73a
Middle	24.58 ± 13.27a	3.77 ± 4.16a
Upper	18.42 ± 9.96b	3.13 ± 3.47a

Means (±SE) within a column followed by the same letters are not significantly different ($P < 0.05$, LSD test). Means displayed are back-transformed from log transformed means used in the statistical analysis. Significance is determined by standard error of the difference between means.

Table 5. Comparison of imidacloprid and olefin concentrations in foliage in each canopy stratum each year post-treatment four to seven years after imidacloprid treatment.

Years Post-Treatment	Canopy Stratum	Imidacloprid Concentration* (ppb)	Olefin Concentration (ppb)
4	Upper	41.58 ± 23.95	8.61 ± 9.84a
	Middle	43.02 ± 24.78	5.59 ± 6.43ab
	Lower	38.68 ± 22.29	5.32 ± 6.13ab
5	Upper	16.70 ± 9.22	4.10 ± 4.59ab
	Middle	19.39 ± 10.70	5.21 ± 5.80ab
	Lower	18.95 ± 10.45	4.60 ± 5.13ab
6	Upper	22.57 ± 12.43	2.02 ± 2.32cd
	Middle	29.97 ± 16.49	3.14 ± 3.53bef
	Lower	25.25 ± 13.90	3.31 ± 3.72bef
7	Upper	7.32 ± 4.26	1.30 ± 1.58df
	Middle	14.58 ± 8.42	2.20 ± 2.59bce
	Lower	8.65 ± 5.02	1.57 ± 1.88cde

Means (±SE) within a column followed by the same letters are not significantly different ($P < 0.05$, LSD test). Means displayed are back-transformed from log transformed means used in the statistical analysis. Significance is determined by standard error of the difference between means.* No significant interaction between years since treatment and strata for imidacloprid concentrations was detected.

concentrations in the lower and middle strata of the canopy six and seven yr post-treatment were not significantly different ($P > 0.05$, LSD test); however, the upper strata had significantly lower concentrations than the lower and middle strata six yr post-treatment ($P < 0.05$, LSD test). The upper stratum six yr post-treatment and the lower and upper strata seven yr post-treatment had significantly lower olefin concentrations than four and five yr post-treatment ($P < 0.05$). Distribution of olefin in the canopy is similar among strata in more recently treated trees. Hemlocks six and seven yr post-treatment experienced lower concentrations of olefin in the upper strata. However, higher concentrations of olefin in the upper and middle strata compared to the lower stratum have been observed (Coots et al. 2013). Overall trends in the data indicate lower olefin concentrations as years since imidacloprid treatment increase.

Concentrations of olefin exceeded the olefin 15 d LC_{50} (6 ppb) for HWA in the upper stratum four yr post-treatment, and all other strata four to seven yr post-treatment experienced less than 6 ppb of olefin in the foliage. Concentrations of both imidacloprid and olefin in most of the canopy strata four to seven yr post-treatment are below the 20 d and 15 d, respectively, LC_{50} for HWA.

Conclusions

In conclusion, imidacloprid and three of its metabolites (olefin, 5-hydroxy, and dihydroxy) were present in hemlock foliage collected from trees four to seven yr after those trees received one imidacloprid basal drench treatment. A low percentage of branchlets contained five-hydroxy and dihydroxy (1.3% and 11.7%, respectively), and the metabolites are not considered to be a contributing factor to effective chemical suppression of HWA four to seven yr

post-treatment. A wide range of imidacloprid and olefin concentrations was documented in foliage, especially four yr post-treatment. Average imidacloprid concentrations were below the 15 and 20 d LC_{50} for HWA four to seven yr post-treatment. Average olefin concentrations were above the 15 d LC_{50} for HWA four yr post-treatment. Over time the concentrations of imidacloprid and olefin decreased. Concentrations in 2013 were lower than those in 2012, and this reduction was observed at each site. Higher concentrations of imidacloprid were present in the middle stratum of the hemlock canopy. While olefin concentrations among strata varied six and seven yr post-treatment, no differences in olefin concentrations among canopy strata four and five yr post-treatment were detected.

Knowledge of the persistence of imidacloprid and olefin and their possible combined additive effect can be used to extend treatment efficacy for longer time periods and facilitate better use of imidacloprid treatments in HWA integrated pest management programs. Treating hemlock trees less often offers HWA management programs both financial and environmental benefits. Financial resources can be saved by treating trees less often, while adding imidacloprid to the forest system less frequently reduces the risk of potential non-target impacts.

CHAPTER III. ASSESSING RELATIONSHIPS BETWEEN TREE DIAMETER AND LONG-TERM PERSISTENCE OF IMIDACLOPRID AND OLEFIN TO OPTIMIZE IMIDACLOPRID TREATMENTS OF EASTERN HEMLOCK

This chapter is revised based on a paper published by Elizabeth Benton, Jerome Grant, Jesse Webster, Rebecca Nichols, Rich Cowles, Anthony Lagalante, and Carla Coots:

Benton, E. P., J. F. Grant, R. S. Cowles, R. J. Webster, R. J. Nichols, R. S. Cowles, A. F. Lagalante, and C. I. Coots. 2016. Assessing relationships between tree diameter and long-term persistence of imidacloprid and olefin to optimize imidacloprid treatments on eastern hemlock. For. Ecol. Manag. 370: 12-21.

My contributions to this paper include (1) reviewing pertinent literature, (2) designing and conducting experiments, (3) processing, analyzing, and interpreting data, and (4) the majority of the writing.

Abstract

Hemlock woolly adelgid (HWA), *Adelges tsugae* (Annand), has caused widespread eastern hemlock mortality in the eastern U.S. HWA was first documented in Great Smoky Mountains National Park (GRSM) in 2002. Once documented, GRSM implemented an aggressive integrated pest management (IPM) program. As a part of this IPM strategy, systemic imidacloprid treatments have been widely used to preserve the Park's hemlock resources. A retrospective study was conducted in cooperation with GRSM to examine the long-term effectiveness of imidacloprid treatments on different size hemlock trees. Of particular interest is olefin, a metabolite of imidacloprid, which is greater than 15 times more toxic to HWA than imidacloprid. The concentrations of imidacloprid and olefin were assessed in hemlock branchlets four to seven years post-imidacloprid treatment. Samples were collected from three strata of the canopy from each of four size classes (30, 45, 61, and 76 cm DBH).

Imidacloprid and olefin were present in all size classes four to seven years after a single imidacloprid treatment. Hemlocks from the 61 and 76 cm size classes exhibited higher imidacloprid and olefin concentrations than the 45 cm hemlocks when larger hemlocks were

given high-dose treatments. When all hemlocks were given low-dose treatments, 61 cm hemlocks had lower concentrations of olefin, but no differences in imidacloprid concentrations among size classes were detected. Olefin concentrations were higher in high-dose compared to low-dose treatments in 76 cm hemlocks. A significant linear relationship exists between the concentration of imidacloprid and olefin in individual branchlets.

A model was developed to optimize the dose of imidacloprid based on the diameter of hemlock trees. Use of this model will result in smaller (< 30 cm) and larger (> 63 cm) hemlocks receiving lower doses of imidacloprid with expected HWA suppression numerous years after treatment. Information obtained from this study can assist resource managers in developing and modifying HWA suppression programs to maintain low HWA populations while reducing the amount of imidacloprid applied to individual hemlocks. Optimal dosing with pesticides based on specific diameter in hemlock systems may result in more environmentally and economically sustainable hemlock management programs.

Introduction

Natural habitats are threatened by exotic and invasive pest species (Vitousek et al. 1996, 1997, Mack et al. 2000). The encroachment of exotic species in a habitat can cause structural and functional changes to occur in natural systems (Castello et al. 1995, Liebhold et al. 1995, Ellison et al. 2005). This phenomenon is not only an ecological process, but an economic one as well (Liebhold et al. 1995). Every year in the United States exotic species in forests cause more than 2 billion dollars in economic impact due to control expenses and product losses (Pimentel et al. 2000). In the last 100 years, many tree species in eastern forests of the United States have

been negatively affected by feeding activity or pathogen transmission by nonnative insect pests. Some species that have either been lost or have suffered massive declines in abundance include: American beech (*Fagus grandifolia* Ehrh.), American chestnut (*Castanea dentata* [Marsh.] Borkh.), eastern hemlock (*Tsuga canadensis* [L.] Carriére), Carolina hemlock (*Tsuga caroliniana* Engelm.), elm (*Ulmus* spp.), and oak (*Quercus* spp.) (Liebhold et al. 1995).

Eastern hemlock populations have declined due to hemlock woolly adelgid, *Adelges tsugae* (Annand) (HWA) (Hemiptera: Adelgidae). The range of eastern hemlock spans much of the eastern United States, from New England south to Georgia and the Atlantic coast west to Wisconsin (USDA Forest Service 1990). Eastern hemlock has a profound influence on the structure and function of its surrounding ecosystem and is an important foundation species in southern Appalachian forests (Ellison et al. 2005). Hemlock is a slow-growing species that inhabits a distinctive ecological niche (Orwig and Foster 1998, Ward et al. 2004). As a shade-tolerant conifer, eastern hemlock plays a vital ecological role in southern Appalachian forests, which cannot be filled by any other native evergreen tree species (Orwig and Foster 1998, Ward et al. 2004). As a low-growing evergreen, hemlock foliage is a source of food for deer in the winter (Lapin 1994) and provides habitat for many bird species (Tingley et al. 2002). Eastern hemlock systems have diverse arthropod communities, with greater than 400 insect species associated with them (Wallace and Hain 2000, Buck et al. 2005, Lynch et al. 2006, Dilling et al. 2007, 2009, Coots et al. 2012). Hemlocks regulate stream water temperatures, and streams associated with hemlock have higher aquatic macroinvertebrate species richness compared to streams in hardwood-dominated watersheds (Snyder et al. 2002, Webster et al. 2012). Many

species depend on eastern hemlock and are being negatively impacted by the loss of this foundation species.

Hemlock woolly adelgid, which is currently present in 19 states (USDA Forest Service 2015b), has caused a drastic reduction in the hemlock population. Depletion of carbohydrates, reduction of photosynthetic ability, reduction in growth, and initiation of a hypersensitive response occurs in hemlocks due to HWA feeding (Young et al. 1995, Radville et al. 2011, Gonda-King et al. 2014, Nelson et al. 2014), eventually leading to hemlock mortality. Tens of millions of dollars have been invested in programs to suppress HWA and preserve hemlock resources (Aukema et al. 2011).

Eastern hemlock is less resistant to HWA than many other hemlock species (Lagalante and Montgomery 2003), and native predator communities do not cause sufficient HWA population suppression (McClure 1987, Wallace and Hain 2000). As a result, insecticides, especially imidacloprid, are a key component of many HWA management programs. Imidacloprid is a neonicotinoid insecticide that affects the central nervous system of insects to cause eventual termination of nerve signals (Nauen and Bretschneider 2002). Some of the metabolites of imidacloprid also have insecticidal properties (Nauen et al. 1998). Of particular importance in HWA control is the insecticidal metabolite imidacloprid-olefin, hereafter referred to as olefin (Coots 2012, Coots et al. 2013, Mayfield et al. 2015, Benton et al. 2015). Olefin is over 15 times more toxic to HWA than imidacloprid based on laboratory dose-response assays (Coots 2012). The persistence of imidacloprid and olefin in hemlocks can provide long-term presence of insecticidal compounds in hemlock foliage for the suppression of HWA (Cook 2008, Coots 2012, Coots et al. 2013, Benton et al. 2015).

The goal of this study is to provide comprehensive information regarding the persistence of imidacloprid and olefin in different size classes of hemlocks (measured as the diameter at breast height [DBH]) for use in forest health management decisions. The current study, which is part of a larger retrospective analysis conducted in collaboration with Great Smoky Mountains National Park (GRSM, also hereafter referred to as the Park), assessed differences in the concentrations of imidacloprid and olefin among hemlocks of different DBH size classes and the distribution of imidacloprid and olefin in the lower, middle, and upper strata of their canopies. In addition, this study explores the variation in imidacloprid and olefin concentrations in low dose imidacloprid treatments among different size hemlocks and both low and high dose imidacloprid treatments in large (76 cm DBH) hemlocks. It is anticipated that the treatment guidelines developed from this information will help to optimize imidacloprid treatments for the suppression of HWA in mixed size hemlock stands.

Materials and Methods

Site/Tree Selection. Study sites were selected based on information provided by Park personnel, the GRSM hemlock treatment database, and site visits. Geographic location, forest composition (hemlock dominant or co-dominant), and imidacloprid treatment history were also considered. Imidacloprid basal drench treatments were applied to hemlocks at all selected sites prior to a drought that occurred in the southern Appalachians in the summer of 2007, thus all hemlocks experienced similar water stress conditions. Hemlocks, depending on the site, were treated with imidacloprid four to seven years prior to sample collection.

Eastern hemlocks (*n* = 137) were selected at three sites (Anthony Creek [35° 35.682 N, 83° 45.845 W], Hesse Creek [35° 40.190 N, 83° 52.126 W]), and Roostertown Road [35° 46.676 N, 83° 14.219 W]) on the western side of GRSM and one site on private land (Mountain Homes, Inc. [35° 40.574 N, 83° 52.144 W]) located adjacent to the western border of the Park (Table 6, Figure 10). Hemlocks at Anthony Creek, Hesse Creek, and Mountain Homes were sampled during the winters of 2011/2012 and 2012/2013. However, Roostertown Road was only sampled during the winter of 2011/2012.

Hemlocks from Mountain Homes (*n* = 33 trees, 17 ha, elevation: 350 m) received imidacloprid treatments in December 2007, 4 yr before sampling was initiated (Table 6). Imidacloprid treatments were applied to hemlocks at Hesse Creek (*n* = 35 trees, 6 ha, elevation: 311 m) and Roostertown Road (n = 35 trees, 1.6 ha, elevation: 548 m) in late 2006 and early 2007, respectively, 5 yr before the first samples were collected. Hemlocks at Anthony Creek (*n* = 34 trees, 21 ha, elevation: 671 m) were treated in late 2006, approximately 6 yr before samples were collected. The height and DBH of hemlocks ranged from 18.3 – 36.6 m and 28 – 79 cm, respectively. Hemlocks were divided into four DBH size classes, 30.4, 45.7, 61.0, 76.2 cm DBH (hereafter referred to as 30, 45, 61, and 76 cm). When a hemlock's diameter was within 2.5 cm of each DBH size class value, it was placed within that size class, i.e. hemlocks between 58.5 and 63.5 cm DBH were included in the 61 cm size class. Each site contained 10 hemlocks from each of the 30, 45, and 61 cm size classes. However, the availability of trees in the 76 cm size class was limited. Each site had between three and five 76 cm size class trees. Metal identification tags with tree labels indicating size class and flagging tape were used to identify study trees.

Table 6. Imidacloprid treatment regimen, number of hemlocks sampled at each site, treatment dates, and sampling dates, Great Smoky Mountains National Park, 2011 – 2013.

Site	Treatment Regimen	No. Hemlocks Sampled per Site	Treatment Date[1]	Sampling Date[2]	
Anthony Creek	Low dose[3]	34	August – October 2006	11, 18 January 2012[4]	23, 24 January 2013[5]
Hesse Creek	Standard dose[6]	35	December 2006	25 January, 1 February 2012	7 February 2013
Mountain Homes, Inc.	Standard dose	33	December 2007	19, 24 January 2012	28, 29 January 2013
Roostertown Road	Standard dose	35	February 2007	21, 22 November 2011	N/A

[1]Month and year imidacloprid was applied to soil underneath hemlock trees.
[2]Date branchlet samples were collected for analysis.
[3]All trees received a low imidacloprid dose (0.7 AI/2.5 cm DBH).
[4]First year of sampling
[5]Second year of sampling
[6]Trees smaller than 63.5 cm DBH received a low imidacloprid dose, and trees 63.5 cm and greater DBH received a high imidacloprid dose (1.4 g AI/2.5 cm DBH).

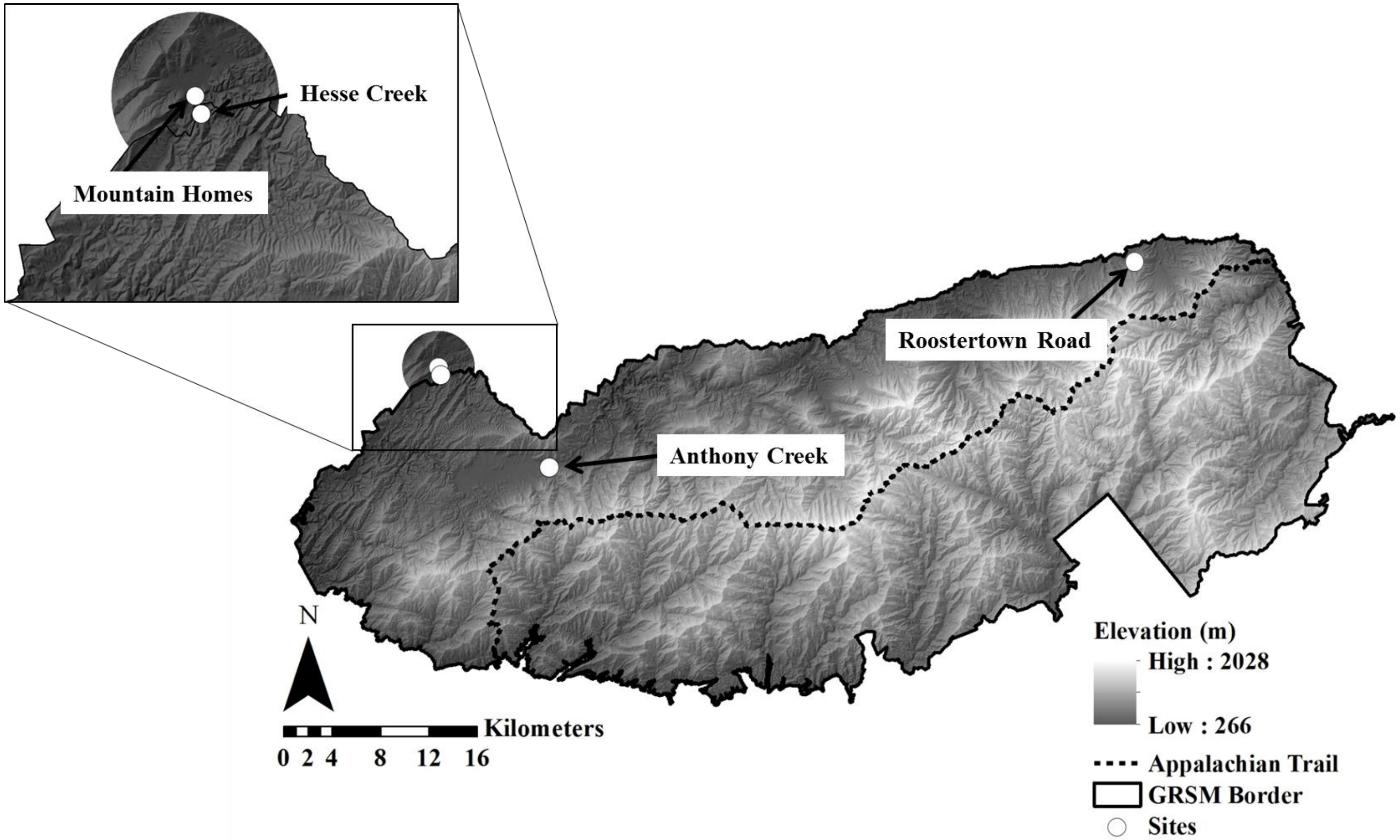

Figure 10. Site locations in Great Smoky Mountains National Park (GRSM).

Insecticide Application. All hemlocks received a basal drench imidacloprid treatment, which entails an imidacloprid suspension being poured into the soil within 0.6 m of the base of each hemlock trunk. Hemlocks in all size classes at Anthony Creek were given a low dose imidacloprid treatment (0.7 g active ingredient [AI]/2.5 cm DBH). Treatments at Hesse Creek, Mountain Homes, and Roostertown Road used two dosages, determined by tree size, as part of the HWA management plan at the sites (Jesse Webster, personal communication). All trees smaller than 63.5 cm DBH received low-dose treatments (0.7 g AI/2.5 cm DBH), and trees 63.5 cm DBH and greater received high-dose treatments (1.4 g AI/2.5 cm DBH). Consequently, the 30 and 45 cm size classes received low-dose treatments, while the 76 cm size class was given high-dose treatments. However, since the dosage change occurs at 63.5 cm, and the DBH size in the 61 cm size class ranges from 58.5 to 63.5 cm, hemlocks in the 61 cm size class from Anthony Creek, Hesse Creek, and Mountain Homes received either high or low dose imidacloprid treatments. Data on the exact dosage applied to individual trees in the 61 cm size class were not available.

Foliage Sampling and Canopy Stratification. Nine branchlet samples (0.5 m in length) were collected from the canopy of each selected hemlock to assess the translocation of imidacloprid and olefin within the canopy of hemlocks in the four DBH size classes. Three branchlet samples were randomly collected from each of three strata (lower third, middle third, and upper third) of the canopy. Because the sites were in remote locations inaccessible by bucket trucks, tree climbers collected the majority of branchlet samples using pole and hand pruners. Some hemlock branches were low enough to allow branchlets to be collected from the ground. Collected branchlet samples were placed in plastic bags (7.5 L), which were placed in cloth

buckets (approximately 19 L) and lowered to the ground. For transport from the field to the laboratory all nine branchlets collected from each hemlock were placed in large plastic bags (49 L). To preserve sample integrity branchlet samples were stored in a walk-in cooler (4°C) for 1-5 d until processed further.

Sample Preparation and Analysis. Branchlets were clipped into small sections (approximately 25 cm in length), and clipped sections from each branchlet were placed in a brown paper bag (2.9 L) to allow air drying of the foliage. Laboratory temperature was maintained at 21°C while samples dried for approximately 2 wk. Once needles could be easily detached from the twigs the samples were ready for further processing. A coffee grinder (Mr. Coffee™, Model IDS77-NP, Rye, NY) was used to pulverize dried needles. Pulverized needle tissue was stored in centrifuge tubes (50 mL) and kept in dark and dry conditions. To extract the imidacloprid and olefin, a 1:10 (needle:solvent) ratio of pulverized needle tissue (1 g) and acetonitrile (10 mL) were mixed in centrifuge tubes (15 mL) and shaken overnight on an orbital bench shaker (New Brunswick Scientific, Edison, NJ; Model G33). The supernatant acetonitrile was analyzed for imidacloprid and its metabolites by liquid chromatography tandem mass spectrometry (LC/MS/MS) at Villanova University. Methods and limits of detection (LOD) of each compound are described in Benton et al. (2015). The detected concentrations of imidacloprid and olefin in $\mu g\ L^{-1}$ from each branchlet were used in the following analyses.

Data Analysis. Excel (Microsoft, Redmond, WA) was used for data storage. All data points below the LOD were entered as a zero, which is a conservative approach to handling data below detectable concentrations. Shapiro-Wilks and Kolmogorov-Smirnov tests were used to test for normality.

Data from Hesse Creek, Mountain Homes, and Roostertown Road were used to analyze differences in concentration of chemicals among size classes as hemlocks at these sites all received a similar treatment regimen of low-dose treatments for smaller hemlocks and high-dose treatments for larger hemlocks. Data used in these analyses were log transformed (ln[x + 0.1]). Because larger hemlocks at Anthony Creek were given low-dose treatments, data from that site were not used in this analysis. Mixed model ANOVA and least significant difference (LSD) mean separation procedure were used to analyze chemical concentration data using the GLIMMIX procedure in SAS (SAS Institute 2008). This analysis was conducted to determine differences among size classes (30, 45, 61, and 76) and among canopy strata (lower, middle, upper) with year, site, years since treatment, year × site, and tree × sampling year × site × size as random effects.

Separate mixed model ANOVA analyses were conducted to determine differences among size classes of hemlocks receiving low-dose treatments and to assess high- and low-dose treatments in large (76 cm) hemlocks. The low-dose treatment analysis used data for all hemlocks at Anthony Creek collected in 2012 and 2013 with year, strata, and tree × size × year as random effects. Data for 76 cm DBH hemlocks from all sites, including Anthony Creek, are included in the low and high dosage comparison. The dosage analysis used year, site, years since treatment, year × site, and tree × year × site as random effects. These analyses from low-dose data collected from Anthony Creek are considered preliminary due to the high hemlock mortality that occurred in large hemlocks at the site.

Data from Hesse Creek, Mountain Homes, and Roostertown Road were used to produce a regression to determine the relationship between the concentration of imidacloprid and olefin

within hemlock branchlets, with imidacloprid concentration as the independent variable and olefin concentration as the dependent variable. Data used in the regression were $\log_{10}$ transformed; residuals from regression analyses indicate that these data fit a log-normal distribution. Samples with olefin concentrations below the LOD were not included in the regression analysis, as the concentrations for those samples were unknown.

A model to optimize imidacloprid dosage relative to tree diameter was developed through several steps. The regression model for the relationship between imidacloprid and olefin residues was combined with published data for the toxicity to HWA of these insecticidal compounds (Coots 2012), to determine a target concentration of insecticide for treated trees. The measured mean values for residues found in the 30 and 45 cm size class trees were used to fit a model to describe the change in residue levels as a function of DBH. The variation in the concentration around the mean was used to adjust the target concentration so that 85% of branch samples would be predicted to result in 80% or greater mortality. Finally, a known treatment dosage resulting in near-optimal concentrations of insecticides was adjusted by our model describing the size vs. residue relationship to give a simple equation to calculate the optimal dosage for any size of hemlock tree. The equation was validated for larger DBH trees using data from low-dose treated 76 cm hemlocks from Anthony Creek.

Results

Imidacloprid Concentration: Size Classes and Strata. Imidacloprid concentrations among hemlock size classes were significantly different ($F = 10.55$; df = 3, 164; $P < 0.001$), and significant differences among canopy strata were detected ($F = 6.16$; df = 2, 1360; $P = 0.002$).

However, a significant interaction between hemlock size class and canopy strata ($F = 5.63$; df = 6, 1360; $P < 0.001$) was detected. Imidacloprid concentrations in the lower and middle strata of the 61 cm hemlocks and the lower stratum of the 76 cm hemlocks were greater than all strata in the 30 and 45 cm hemlocks ($P < 0.05$, LSD test) (Table 7). Each size class varied in the distribution of imidacloprid within the canopy strata. The middle canopy stratum of 30 cm hemlocks had significantly higher imidacloprid concentrations than the lower stratum ($P < 0.05$, LSD test). Imidacloprid concentrations among canopy strata in 45 cm hemlocks did not significantly differ ($P > 0.05$, LSD test). The lower stratum of 61 cm hemlocks had a significantly higher concentration of imidacloprid than the middle and upper strata, and the middle and lower strata of 76 cm hemlocks had higher imidacloprid concentrations than the upper stratum ($P < 0.05$, LSD test). No consistent and significant trends were observed within each hemlock size class. However, a pattern is present between the 30 and 45 cm hemlocks and the 61 and 76 cm hemlocks. In general, concentrations of imidacloprid are greater in the middle and upper canopy strata of smaller trees, compared with their lower canopy. In contrast, imidacloprid concentrations are greater in the lower canopy of larger trees, most of which received high-dose imidacloprid treatments 4 – 6 yr prior to sampling. The LC_{50} of imidacloprid for HWA has been documented as 112 and 300 µg L^{-1} in 15 d and 20 d, respectively, laboratory dose-response assays (Cowles et al. 2006, Coots 2012). Additionally, HWA suppression has been observed in forest settings when imidacloprid concentrations were in excess of 120 µg L^{-1} in hemlocks 2 yr post-treatment (Cowles et al. 2006). The highest average concentration of imidacloprid in the current study was 60.2 µg L^{-1}, observed in the lower stratum of the 61 cm

Table 7. Comparison of imidacloprid and olefin concentrations in foliage in each canopy stratum in four hemlock size classes, Great Smoky Mountains National Park, 4 – 6 years post-treatment.

Size Class (cm DBH)	Canopy Stratum	n	Imidacloprid Concentration		Olefin Concentration	
			ln transformed[1]	$\mu g\ L^{-1}$ [2]	ln transformed	$\mu g\ L^{-1}$
30	Upper	150	3.26 ± 0.81cdef	25.86	1.67 ± 1.28bc	5.24
	Middle	150	3.34 ± 0.81cde	28.06	1.57 ± 1.27bc	4.72
	Lower	150	3.11 ± 0.81fg	22.39	1.14 ± 1.28def	3.03
45	Upper	150	2.94 ± 0.81fg	18.82	0.81 ± 1.28ef	2.14
	Middle	150	2.99 ± 0.81efg	19.82	0.66 ± 1.28f	1.83
	Lower	150	2.78 ± 0.81g	16.04	0.67 ± 1.28f	1.86
61	Upper	150	3.63 ± 0.81bcd	37.55	1.52 ± 1.28cde	4.48
	Middle	150	3.81 ± 0.81b	45.16	1.98 ± 1.28b	7.15
	Lower	150	4.09 ± 0.81a	60.19	2.41 ± 1.28a	11.05

Table 7. Continued.

Size Class (cm DBH)	Canopy Stratum	n	Imidacloprid Concentration		Olefin Concentration	
			ln transformed[1]	μg L^{-1} [2]	ln transformed	μg L^{-1}
76	Upper	63	3.24 ± 0.83defg	25.54	1.86 ± 1.32abcd	6.35
	Middle	63	3.70 ± 0.83abc	40.38	2.41 ± 1.32abc	11.07
	Lower	63	3.84 ± 0.83ab	46.66	2.41 ± 1.32abc	11.09

[1]Means (±SE) of natural log transformed data within a column followed by the same letters are not significantly different (*P* > 0.05, LSD test). Significance is determined by standard error of the difference between means.
[2]Means in μg L^{-1} are back-transformed from log transformed means used in the statistical analysis.

size class. All observed average concentrations of imidacloprid from each stratum of each hemlock size class are below both the 15-d and 20-d LC_{50} for HWA.

Olefin Concentration: Size Classes and Strata. A significant difference in olefin concentrations among hemlock size classes was detected (F = 6.43; df = 3, 166; P < 0.001). Significant differences in olefin concentrations among canopy strata were not observed (F = 1.96; df = 2, 1360; P = 0.142) (Table 7), however a significant interaction between olefin concentrations among hemlock size class and canopy strata was detected (F = 5.52; df = 6, 1360; P < 0.001) (Table 7). All canopy strata of 76 cm hemlocks, the middle and lower strata of 61 cm hemlocks, and the middle and upper strata of 30 cm hemlocks are significantly higher than all strata of 45 cm hemlocks (P < 0.05, LSD test). No consistent significant trends were observed within each hemlock size class. Significant differences in olefin concentrations among strata in both the 45 and 76 cm hemlocks were not detected (P > 0.05, LSD test). The upper and middle strata of 30 cm hemlocks had significantly higher olefin concentrations than the lower stratum (P < 0.05, LSD test).   All strata in 61 cm hemlocks were significantly different. The upper stratum had the lowest concentrations of olefin, followed by the middle stratum, while the lower stratum had the highest concentration of olefin (P < 0.05, LSD test). Similar to the observed pattern with imidacloprid, olefin concentrations are higher in the upper canopy of smaller trees as compared to the lower canopy, and concentrations increase from the upper to the lower canopy of larger trees.

While no consistent significant trends in imidacloprid and olefin concentration among individual hemlock size classes were detected, some similarities between imidacloprid and olefin concentrations were found within the canopy strata of each size class. Imidacloprid and olefin

concentrations in 30 cm hemlocks were lowest in the lower stratum. No differences in imidacloprid and olefin concentrations among strata in 45 cm hemlocks were observed. Imidacloprid and olefin concentrations in 61 cm hemlocks were highest in the lower stratum and lowest in the upper stratum. The lower and middle strata of 76 cm hemlocks contained higher imidacloprid concentrations. However, olefin concentrations did not differ among strata in 76 cm hemlocks.

The 15-d LC_{50} of olefin for HWA is 6 µg L^{-1} (Coots 2012). The concentrations of olefin in each stratum of the 30 and 45 cm hemlocks were below the LC_{50} for HWA. The mean olefin concentrations in all strata of 76 cm hemlocks and the lower and middle strata of 61 cm hemlocks were greater than the LC_{50}. Overall olefin concentrations in the 61 and 76 cm hemlocks are sufficiently high for HWA population suppression numerous years after treatment. However, it is important to note that all 76 cm hemlocks and a portion of the 61 cm hemlocks received a high dose imidacloprid treatment 4 – 6 yr prior to sampling.

Low Dose Comparison Among Hemlock Size Classes. The 76 cm hemlocks had the highest imidacloprid concentrations, while the lowest imidacloprid concentrations were found in 61 cm hemlocks. However, significant differences in imidacloprid concentrations among size classes of hemlocks that received low-dose treatments were not detected ($F = 2.50$; df = 3, 64; $P > 0.05$) (Table 8). Olefin concentrations among size classes of hemlocks that received low-dose treatments were significantly different ($F = 7.44$; df = 3, 64; $P < 0.001$). The highest olefin concentration was detected in 45 cm hemlocks. The lowest concentration was detected in 61 cm hemlocks, which had significantly lower concentrations than all other size classes ($P < 0.05$,

Table 8. Low dose comparison of imidacloprid and olefin concentrations in foliage of four hemlock size classes at the Anthony Creek site, Great Smoky Mountains National Park, 6 – 7 years post-treatment.

Size Class (cm DBH)	N	Imidacloprid Concentration		Olefin Concentration	
		log transformed[1]	µg L^{-1} [2]	log transformed	µg L^{-1}
30	20	2.49 ± 0.91a	11.92	0.62 ± 1.23b	1.77
45	20	2.81 ± 0.91a	16.58	1.72 ± 1.23a	5.50
61	20	2.33 ± 0.91a	10.19	-0.34 ± 1.23c	0.61
76	9	3.36 ± 0.94a	28.80	1.01 ± 1.28ab	2.36

[1]Means (±SE) of natural log transformed data within a column followed by the same letters are not significantly different ($P > 0.05$, LSD test). Significance is determined by standard error of the difference between means.
[2]Means µg L^{-1} are back-transformed from log transformed means used in the statistical analysis.

LSD test). Olefin concentrations were significantly higher in 45 cm hemlocks compared to 30 cm hemlocks, and olefin concentrations in 76 cm hemlocks were not significantly different from either 30 or 45 cm hemlocks ($P > 0.05$, LSD test). All imidacloprid and olefin concentrations from low dose imidacloprid treatments were below the imidacloprid 15 and 20-d LC_{50} and olefin 15-d LC_{50} for HWA from foliage of trees that were sampled 6 and 7 yr post-treatment.

The low-dose comparison among hemlock size classes and the high- and low-dose comparison in large hemlocks, which used data from Anthony Creek, are considered preliminary analyses. HWA populations were high on hemlocks at Anthony Creek at the time of imidacloprid application. Most of the larger trees did not survive, and so those surviving hemlocks used in the analyses represent a biased sample. Among the larger group initially treated, these were the only trees that translocated enough imidacloprid and olefin to have suppressed the HWA populations and survived.

Low and High Dose Comparison in Large Hemlocks. Imidacloprid concentrations in foliage of 76 cm hemlocks that received low compared to high-dose treatments were not significantly different ($F = 0.09$; df = 1, 240; $P > 0.05$), and all imidacloprid concentrations were below the LC_{50} for HWA. A significant difference was detected in olefin concentrations ($F = 4.50$; df = 1, 240; $P < 0.035$) (Table 9). Olefin concentrations in high-dose treatments were significantly greater than low-dose treatments ($P < 0.05$, LSD test). The foliage of hemlocks given high-dose treatments contained olefin concentrations more than four times greater than those in low-dose treatments. Olefin concentrations in foliage from high-dose treatments were almost twice the 15-d LC_{50} for HWA.

Table 9. Comparison of imidacloprid and olefin concentrations in foliage of 76 cm DBH hemlocks that received either high or low-dose treatments, Great Smoky Mountains National Park.

Imidacloprid Dose	Imidacloprid Concentration		Olefin Concentration	
	log transformed[1]	$\mu g\ L^{-1}$ [2]	log transformed	$\mu g\ L^{-1}$
Low[3]	3.40 ± 0.55a	30.01	1.01 ± 0.88b	2.65
High[4]	3.56 ± 0.43a	35.12	2.45 ± 0.77a	11.52

[1]Means (±SE) of natural log transformed data within a column followed by the same letters are not significantly different ($P > 0.05$, LSD test). Significance is determined by standard error of the difference between means.
[2]Means in $\mu g\ L^{-1}$ are back-transformed from log transformed means used in the statistical analysis.
[3]Low dose treatments were assessed 6-7 yr post-treatment.
[4]High dose treatments were assessed 4-6 years post-treatment.

Relationship Between Imidacloprid and Olefin Concentrations in Branchlets. A positive log-log linear relationship was found between the concentration of imidacloprid and the concentration of olefin in hemlock branchlets ($P < 0.001$) (Figure 11). A slope of 0.955 indicates that the observed concentrations of imidacloprid and olefin have nearly a linear relationship, rather than involving more complicated enzyme kinetics. The linear regression model describing the relationship between concentrations (C) of imidacloprid and olefin (Equation 1) accounts for 88% of the observed variation in the data ($R^2 = 0.88$).

Equation 1: $\log(C_{\text{olefin}}) = \left[0.955 \times \log\left(C_{\text{imidacloprid}}\right)\right] - 0.178$

Optimization of Imidacloprid Dosage Relative to Tree Diameter. The concentration of olefin found in hemlock foliage was greater than one-fifteenth the concentration of imidacloprid (Table 7), signifying that of these two insecticidal compounds, the toxic effects of the olefin would predominate (Coots 2012). Therefore, an optimum management strategy should be to apply imidacloprid at a concentration that can be expected to subsequently convert to the olefin, so that a targeted concentration of olefin sufficient to provide multiple years of control will be reached. An equation to optimize the dosage of imidacloprid relative to the DBH of hemlocks was developed by using a target imidacloprid concentration and known concentrations of imidacloprid in 30 and 45 cm hemlocks numerous years after receiving low-dose treatments to determine variables of a non-linear model. The concentration of olefin found in hemlock tissue is closely related to the concentration of imidacloprid found in the same branchlet. The regression between imidacloprid and olefin concentrations indicated that ~88% of the experimental variation was accounted for, when both variables had first been log-transformed. The regression

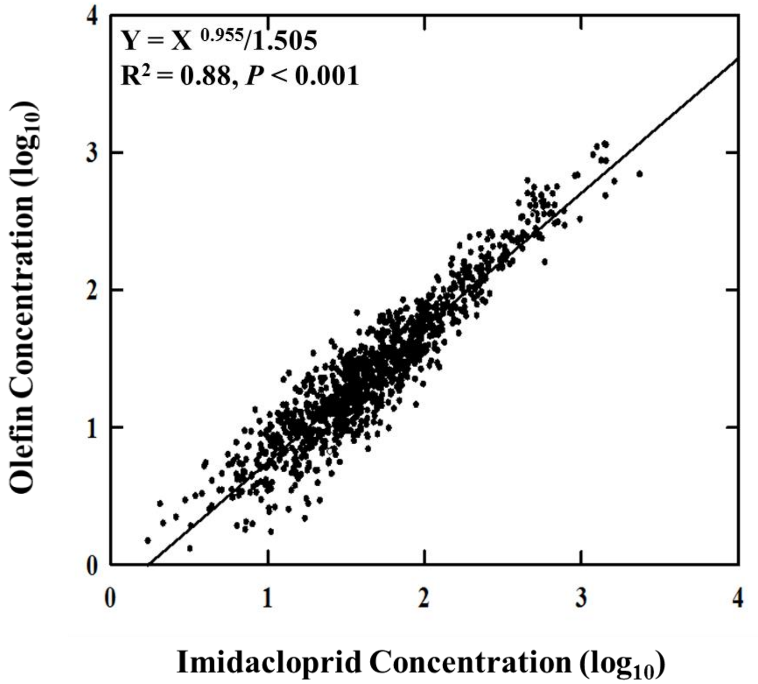

Figure 11. Log-log relationship between imidacloprid and olefin concentrations (µg L^{-1}) in hemlock branchlets.

model, based on log-transformed data, generates Equation 2, when both sides of the linear regression equation are exponentiated.

Equation 2: $$C_{\mathrm{olefin}} = \frac{(C_{\mathrm{imidacloprid}})^{0.955}}{1.505}$$

A target concentration of olefin was determined to be 7 µg L^{-1}, the LC_{80} for HWA as determined by Coots (2012). This olefin concentration, in turn, would result from an imidacloprid concentration of 11 µg L^{-1}, as determined by Equation 2. However, adjustment of this concentration upwards ensures that a larger proportion than 50% of the branchlets would attain at least 80% mortality. By targeting the concentration upwards by one standard deviation from the mean, 85% of the branchlets would exceed the target for olefin. The standard deviations of the log-transformed, mean imidacloprid concentrations for 30 and 45 cm size classes are 0.41 and 0.47, respectively. The pooled standard deviation is 0.44, which when added to the log of the mean targeted concentration (11 µg L^{-1}), and reverse transformed, produces the new target concentration of imidacloprid: 32.2 µg L^{-1}.

The mean and standard deviations for the imidacloprid concentrations for the 30 and 45 cm DBH trees sampled five years post-treatment were 1.60 ± 0.41, and 1.51 ± 0.47, with $\log_{10}$-transformed data. The back-transformed means, 39.5 and 32.5, respectively, were used to fit a non-linear curve relating tissue concentration to DBH. It is known from previous work (Cowles 2009) that the concentration in foliage must be inversely proportional to some function of the tree diameter. Several candidate models were tested, with Equation 3 providing the best fit.

Equation 3: $$C_{\mathrm{imidacloprid}} = m \times \left[\frac{1}{\log(DBH)^{b}}\right]$$

Two data points, 30 and 45 cm hemlocks, have known values for mean imidacloprid concentration in foliage, DBH, and low-dose treatment rates. These data points were used to fit a

candidate model for optimizing imidacloprid doses by hemlock DBH. Excel was used to determine which values were needed for the m and b parameters to obtain the expected concentrations of imidacloprid in Equation 3. The result was Equation 4.

Equation 4: $$C_{\text{imidacloprid}} = 78.6 \times \left[\frac{1}{\log(DBH)^{1.745}}\right]$$

A good validation of this model is provided by comparing the prediction for 76 cm DBH trees, which is 26.1 µg L^{-1}, to the measured concentration (28.8 µg L^{-1}) for the low-dose treated trees of this size. The predicted value is less than 1.5 times the standard error of the mean [the pooled standard error of the mean imidacloprid concentrations for the smaller diameter trees was 0.0315, for log-transformed data], signifying that the model provides a reasonably accurate fit even when extrapolating to the 76 cm DBH trees.

Equation 4 was then used to calculate the optimal dosage for any size of tree. The following equation calculates the proportional change in dose as diameter changes, based on the fact that the 45 cm hemlocks with a dosage of 0.7 g AI/2.5 cm DBH produced tissue concentrations close (32.5 µg L^{-1}) to our target value of 32.2 µg L^{-1} for imidacloprid.

Equation 5: $$\text{optimum dose} = 0.7 \times \left[\frac{\log(DBH)^{1.745}}{\log(45)^{1.745}}\right]$$

Equation 5 simplifies to Equation 6. This equation provides the dose per 2.5 cm DBH for imidacloprid active ingredient, based on a tree's DBH measured in cm.

Equation 6: $$\text{optimum dose} = 0.3 \times \log(DBH)^{1.745}$$

Optimal doses for hemlock DBH can then be added to a DBH tape or Biltmore stick for convenient use in field applications (Table 10). The optimal doses of imidacloprid for smaller (< 30 cm) and larger (> 63 cm) hemlocks are less than the current method of increasing imidacloprid doses at 63.5 cm and the optimization formula developed by Cowles (2009), as can

Table 10. Optimized dosages according to hemlock diameter at breast height (DBH).

Centimeters DBH	Inches DBH[1]	g AI per 2.54 cm DBH[2]	Total g AI per tree
20	7.9	0.500	3.933
30	11.8	0.623	7.358
40	15.7	0.718	11.299
50	19.7	0.795	15.644
60	23.6	0.860	20.321
70	27.6	0.917	25.283
80	31.5	0.968	30.493
90	35.4	1.014	35.925
100	39.4	1.056	41.557

[1] 1 inch = 2.54 cm
[2] The optimum dosage equation [optimum dose – $0.3 \times \log(\mathrm{DBH})^{1.745}$] uses cm DBH to produce g AI per every 2.54 cm DBH.

be observed in Figure 12. Use of the optimization formula would result in a gradual increase in the rate of imidacloprid applied based on hemlock DBH, which translates into less imidacloprid added to the system in mixed DBH hemlock stands.

Discussion

Imidacloprid and olefin concentrations varied among different size hemlocks. The current study is the first study to our knowledge that has determined if differences in concentrations of both imidacloprid and olefin occur among different size classes of hemlocks. Previous studies assessing the translocation of imidacloprid in hemlocks have used hemlocks ranging in size from 5 – 60 cm DBH (Cowles 2009, Joseph et al. 2011a, Coots et al. 2013, Eisenback et al. 2014, Mayfield et al. 2015). These studies have assessed applications of imidacloprid ranging from 0.15 – 1.5 g AI/2.5 cm DBH, to determine spatial and temporal distribution within the canopy, effects of fertilizer and imidacloprid on HWA populations, impacts of low-dose applications on both HWA and biological control beetles, and optimization of imidacloprid dosage. However the current study explores if both imidacloprid and olefin concentrations vary within the strata and DBH size classes.

Variation occurs in imidacloprid and olefin translocation to the lower, middle, and upper canopy strata among hemlocks in different DBH size classes. Different imidacloprid concentrations were detected among hemlock canopy strata. However, results are different from observations in Benton et al. (2015), a study in which many of the same hemlocks were assessed by year since treatment. Hemlocks from Hesse Creek and Mountain Homes were used in both analyses. Hemlocks from Anthony Creek were included in the previous analysis, while

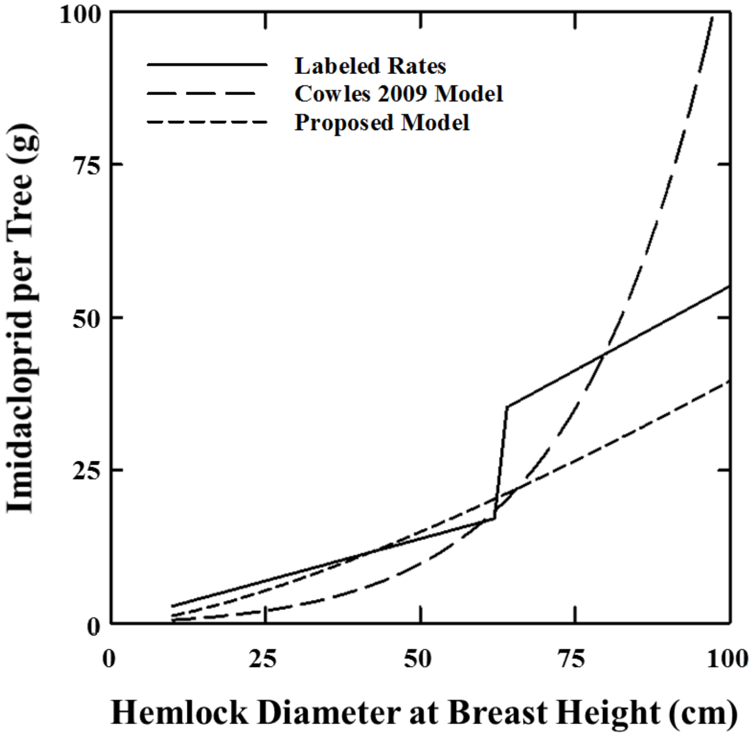

Figure 12. Comparison of amounts of imidacloprid applied per tree according to different dosage models.

hemlocks from Roostertown Road were used in the current study. Imidacloprid concentrations found in the middle stratum were higher than the lower and upper strata (Benton et al. 2015); however that trend was not observed in this study. In addition, Coots et al. (2013) detected the highest concentrations of imidacloprid in the lower stratum of hemlock canopies and higher olefin concentrations in the middle and upper strata. No overall differences in olefin concentrations among canopy strata were detected by Benton et al. (2015). However, differences in olefin concentrations among canopy strata were observed in the current study. Variation in concentrations of imidacloprid and olefin among canopy strata observed in the current study, as well as Coots et al. (2013) and Benton et al. (2015) may be due to site-specific parameters, such as slope, aspect, and rainfall history.

An opposing trend was detected when comparing distribution of imidacloprid and olefin among the strata in the canopy of 30 and 45 cm hemlocks compared to 61 and 76 cm hemlocks. Smaller hemlocks had a trend of higher imidacloprid and olefin concentrations in the upper canopy, while larger hemlocks had a trend of higher concentrations in the lower canopy. The observed pattern may be attributed to the imidacloprid dosage, the size of the live crown in smaller trees compared to larger trees, or the degree of sunlight exposure occurring on the canopy. Smaller trees are likely to have increased shading of the lower canopy, which may cause the lower canopy to transpire less, moving less water, and contributing to lower levels of imidacloprid as compared to the upper canopy.

Translocation of imidacloprid throughout the hemlock canopy occurs via movement through the xylem in the trunk and branches, and eventually the xylem ray parenchyma cells where HWA feed. Mass flow movement of water in the hemlock, and thus imidacloprid, is

highly influenced by tree diameter (Ford et al. 2007, Ford and Vose 2007). The exponential relationship of increased water use with increasing tree diameter has been documented in hemlocks, as well as many other tree species (Meinzer et al. 2005, Ford et al. 2007). It has been suggested that dosages of imidacloprid should increase exponentially with tree DBH to ensure adequate insecticide concentrations in the canopy, and that using the same dosage for all hemlock DBH sizes would result in higher imidacloprid concentrations in smaller trees compared to larger trees (Ford et al. 2007). The increase in dosage in the current study involved doubling the rate of imidacloprid applied per 2.5 cm DBH for trees larger than 63.5 cm. This attempt to adjust the dosage relative to tree diameter is somewhat arbitrary; a closer fit to optimal dosing may be useful for preventing overdosing of small trees, as well as providing dosing precisely calibrated from empirical residue data for larger trees.

Consistency in imidacloprid and olefin concentrations among different DBH size hemlocks when larger trees were given higher imidacloprid doses has not been observed in the current study. When high dose imidacloprid treatments were applied to larger trees, the large hemlocks generally had higher concentrations of imidacloprid and olefin compared to some smaller size classes, which received low-dose treatments. Strata in the 76 cm hemlocks had up to a three-fold increase in olefin concentration (in $\mu g\ L^{-1}$) compared to strata in 45 cm hemlocks. When all hemlocks were given low-dose treatments, no differences in imidacloprid concentrations among hemlock size classes were detected. Olefin concentrations in 76 cm hemlocks were not different than 30 and 45 cm hemlocks. The lower imidacloprid and olefin concentrations observed in 45 cm hemlocks compared to 30 cm hemlocks, both of which were given low-dose treatments, suggest that a dilution factor may occur between the amount of

imidacloprid applied and the increase in the amount of hemlock tissue between these two size classes. A similar trend was observed between the 61 and 76 cm size classes, for the data enumerated in Table 7.

Management decisions regarding treatment doses involve a trade-off between level of HWA suppression and amount of imidacloprid applied to the system for both environmental and financial concerns. A greater than 90% reduction in HWA populations has been observed in hemlocks receiving 0.5 and 0.75 g AI/2.5 cm DBH low-dose treatments three years post-treatment (Cowles 2009). Concentrations of olefin in excess of the 15-d LC_{50} for HWA were present in hemlocks 4 yr post-treatment from the Mountain Homes site, where large hemlocks received high-dose treatments (Benton et al. 2015). High imidacloprid doses (1.5 g. AI/2.5 cm DBH) may result in higher concentrations of imidacloprid and olefin in larger hemlocks, as indicated by the observed higher concentrations in the 61 and 76 cm hemlocks compared to the 45 cm hemlocks. However, HWA management plans can often require treating an entire hemlock stand at the same time due to time and resource constraints. The presence of higher concentrations of imidacloprid and olefin observed in larger hemlocks 4 – 6 years after high-dose treatments may not be necessary, as a hemlock stand may have to be retreated sooner to provide adequate HWA protection for smaller hemlocks receiving low-dose treatments.

Application of imidacloprid cannot exceed 0.45 kg AI/hectare during a year (Bayer 2015). Often all hemlocks in high density or pure hemlock stands cannot be treated during one year without exceeding the pesticide label maximum dosage per hectare. In large HWA management programs, revisiting a site within a few years after initial imidacloprid applications for additional treatments may not be feasible. If high dose imidacloprid applications are given to

larger hemlocks, then smaller trees may not receive treatments to prevent exceeding the maximum rate per hectare. Trade-offs must be made between low and high dose applications to suppress HWA on select larger hemlocks and a greater number of smaller hemlocks in the treatment plan for a site. Imidacloprid and olefin concentrations were not consistently significantly higher in all high dose large hemlock strata compared to all low dose small hemlock strata. In addition, olefin concentrations were not lower in all large size class compared to small size classes when low-dose treatments were used.

Using the knowledge of expected olefin and imidacloprid concentrations in different size hemlocks, paired with the relationship between imidacloprid and olefin concentrations in branchlets has allowed for the development of a formula for optimization of imidacloprid dosage based on the DBH of hemlocks. The total amount of imidacloprid applied per hemlock could be reduced by using the optimal dose equation compared to the current dosage plan and the dosage equation from Cowles (2009). By applying less imidacloprid per tree, optimized for DBH, managers will be able to treat more hemlocks while staying in compliance with label limits. Knowledge of expected imidacloprid and olefin concentrations in different hemlock size classes, when low and high-dose treatments are applied, as well as introduction of a formula to optimize dosage of imidacloprid based on DBH (cm) of hemlocks, can offer resource managers guidance for determining management plans for sites with high hemlock densities.

Conclusions

Differences were detected in both imidacloprid and olefin concentrations among different size hemlocks. Differences varied based on imidacloprid treatment dosage, with larger hemlocks

exhibiting higher concentrations of both chemicals in most canopy strata, as compared to 45 cm hemlocks when high dose imidacloprid treatments were applied to larger hemlocks. In contrast, in the low-dose comparison 45 cm hemlocks had the highest concentration of olefin, however, no difference was detected between 45 and 76 cm hemlocks receiving low-dose treatments. Imidacloprid concentrations among hemlock size classes in the low dose comparison were not significantly different. Olefin concentrations were significantly higher in 76 cm hemlocks that received high-dose treatments in the preliminary low and high dose comparison. While no overall consistent patterns in either imidacloprid or olefin concentrations among all size classes occurred, consistencies were observed between imidacloprid and olefin canopy strata distribution within each size class. In addition, a formula was developed to optimize dosage of imidacloprid based on hemlock DBH. According to the formula, smaller (< 30 cm) and larger (> 63 cm) hemlocks can receive lower dosage imidacloprid applications. Olefin persistence in the foliage is expected to occur in concentrations sufficient to provide > 80% HWA population reduction per generation 4 – 6 yr post-treatment.

Numerous state and federal agencies, as well as private land owners, have developed HWA management programs to preserve hemlock resources. Temporal factors, financial constraints, and desired degree of HWA suppression factor into management decisions regarding imidacloprid dosage and time between treatments. Knowledge of the persistence of imidacloprid and olefin in the canopy strata of various sized hemlocks numerous years post-treatment, as well as a tool to optimize imidacloprid doses based on hemlock DBH, will better guide land managers to maximize use of management program resources to protect eastern hemlocks.

CHAPTER IV. HEMLOCK WOOLLY ADELGID ABUNDANCE AND HEMLOCK CANOPY HEALTH NUMEROUS YEARS AFTER IMIDACLOPRID BASAL DRENCH TREATMENTS: IMPLICATIONS FOR MANAGEMENT PROGRAMS

This chapter is revised based on a paper submitted by Elizabeth Benton, Jerome Grant, Jesse Webster, Richard Cowles, Anthony Lagalante, Rebecca Nichols, and Carla Coots:

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My contributions to this paper include (1) reviewing pertinent literature, (2) designing and conducting experiments, (3) processing, analyzing, and interpreting data, and (4) the majority of the writing.

Abstract

Hemlock woolly adelgid (*Adelges tsugae* [Annand]) (HWA), an invasive insect in the eastern United States, has caused widespread decline of eastern hemlock, *Tsuga canadensis* (L.) Carriére. Imidacloprid basal drench treatments were assessed 4 – 7 yr after a single treatment to determine population suppression of HWA and effects on hemlock canopy health. The effects of site, years post-treatment, and hemlock size classes measured as diameter at breast height (DBH) were evaluated relative to imidacloprid treatment on HWA populations, hemlock live crown ratio, crown density, foliage transparency, and crown dieback. Imidacloprid treatments resulted in low-level HWA populations 7 yr post-treatment. HWA populations increased as yr post-treatment increased. Smaller hemlocks, which were dosed with 0.7 g active ingredient (AI)/2.5 cm DBH, had higher populations of HWA than the largest size class of trees, which were treated at twice that dosage. Suppression of HWA populations while concentrations of imidacloprid and olefin were below the LC_{50} suggests an additive effect of imidacloprid and its olefin metabolite, and compounded mortality over many generations. HWA populations were

too low to have an observable effect on hemlock canopy health, indicating application intervals of up to 7 yr may be adequate to protect hemlocks.

Introduction

Hemlock woolly adelgid (*Adelges tsugae* [Annand]) (HWA) (Hemiptera: Adelgidae) is an invasive insect native to Japan, China, and the Pacific Northwest of the United States, with genetically differentiated populations occurring among these regions (Havill et al. 2006). Although first documented in western North America on western hemlock (*Tsuga heterophylla* [Raf.] Sarg.) (Pinales: Pinaceae) as early as 1922 in British Columbia and soon after in California and Oregon (Annand 1928), western hemlock appears to tolerate HWA feeding. Hemlock woolly adelgid was later documented in the eastern United States from Virginia in 1951 (Stoetzel 2002) with a population later identified as having originated in Japan (Havill et al. 2006). While nine *Tsuga* species occur worldwide, HWA is only a serious pest of eastern hemlock (*Tsuga canadensis* [L.] Carrière) (Pinales: Pinaceae) and Carolina hemlock (*Tsuga caroliniana* Engelm.) (Pinales: Pinaceae). Neither of these hemlock species, which are native to the eastern United States, coevolved with adelgids, so they fare poorly when challenged with *A. tsugae* (McClure et al. 2001). Eastern hemlocks are infested with HWA throughout much of their native range, with the HWA population originating from Japan continuing to expand north and westward (Havill et al. 2006, Lambdin et al. 2006, USDA Forest Service 2013).

Hemlock woolly adelgid is parthenogenic and has two generations each year: the sistens and progrediens generations. Sistens have a longer lifespan, living for approximately 9 mo. They hatch in late spring, enter a dormant phase during summer, and begin feeding during fall.

Progrediens have a shorter, approximately 3 mo, lifespan. Progrediens eggs, laid by the sistens generation, hatch in early spring. Progrediens feed for several months and lay eggs during late spring (Cheah et al. 2004).

Upon hatching, first-instar nymphs (crawlers) locate the base of a hemlock needle where they attach their stylet and feed by withdrawing fluid from the xylem ray parenchyma (Young et al. 1995). This feeding depletes critical storage tissues of hemlock (Young et al. 1995). When carbohydrates and nutrients in the xylem ray parenchyma are depleted, less energy is available for tree growth, defense, metabolism, and reproduction (Shigo 1989). HWA alters the ability of eastern hemlock to photosynthesize (Nelson et al. 2014). Foliage infested with HWA exhibits decreased growth, reduced bud production, and lower water potential (Gonda-King et al. 2014). In addition, HWA infestations on hemlock foliage negatively alter the mechanical properties of needles by reducing mechanical strength and lowering twig flexibility (Soltis et al. 2014). When trees experience heavy infestation levels, little to no new foliage develops, and as the infestation progresses, the trees experience graying of the needles, branch mortality, and an increasingly thinner canopy (Orwig and Foster 1998, Jenkins et al. 1999, McClure and Cheah 1999, Stadler et al. 2005, Eschtruth et al. 2006). Often hemlock mortality occurs in as few as 2 – 4 yr after initial infestation, especially where winter temperatures do not limit HWA survival (McClure 1991, Orwig et al. 2002, Skinner et al. 2003, Nuckolls et al. 2009).

Eastern hemlock is an important component of eastern forests, as it is a slow-growing evergreen species that inhabits a distinctive ecological niche (Orwig and Foster 1998, Ward et al. 2004). As the only shade-tolerant conifer in southern Appalachian forests, eastern hemlock plays a vital ecological role, especially in southern Appalachian forests. Unfortunately, a

suitable native replacement species that can fill the ecological role of eastern hemlock is not available (Orwig and Foster 1998, Ward et al. 2004). Changes observed in hemlock forests as a result of hemlock decline include a diminished canopy which causes greater light infiltration, resulting in a drier forest floor, changes in nitrogen cycling in the soil, and an increase of downed woody debris (Orwig and Foster 1998, Jenkins et al. 1999, Eschtruth et al. 2006, Orwig et al. 2008). Eastern hemlock provides habitat for many vertebrate and invertebrate species (Wallace and Hain 2000, Tingley et al. 2002, Buck et al. 2005, Dilling et al. 2007, 2009, Mallis and Rieske 2011, Coots et al. 2012). The continued decline of eastern hemlock populations will cause cascading biotic and abiotic effects in eastern forests.

Hemlock integrated pest management (IPM) programs employ numerous control tactics to suppress HWA in eastern forests. A multi-faceted program, such as that at Great Smoky Mountains National Park (GRSM), may employ insecticides, such as horticultural oil, insecticidal soap, and the neonicotinoid pesticides imidacloprid and dinotefuran (Webster 2010). Imidacloprid treatments have been highly effective in multi-year HWA suppression (Cowles and Cheah 2002, Cowles et al. 2006, Coots 2012, Coots et al. 2013, Eisenback et al. 2014, Mayfield et al. 2015). The success of imidacloprid is, in part, due to the longevity of the insecticidal metabolite olefin, which is over 14 times more toxic to HWA than imidacloprid (Coots 2012). In addition, IPM programs may include biological control, including release of predatory beetles such as *Sasajiscymnus tsugae* (Sasaji and McClure) (Coleoptera: Coccinellidae) and *Laricobius nigrinus* (Fender) (Coleoptera: Derodontidae) (Webster 2010). Many recent studies have focused on obtaining a balance between chemical and biological options with the goal of maintaining HWA populations that are sufficient to support predator populations, but low

enough to not negatively affect hemlock health (Eisenback et al. 2010, 2014, Joseph et al. 2011a,b, Mayfield et al. 2015). The choice of HWA control tactics employed by land managers is largely driven by available funds and target program outcomes.

The current study, conducted in collaboration with GRSM, is part of a comprehensive retrospective project to assess the HWA IPM program at GRSM. Specifically the longevity and efficacy of imidacloprid basal drench treatments in mixed DBH (diameter at breast height) size hemlock stands were assessed 4 – 7 yr after a single imidacloprid treatment to produce research-based guidance on the HWA IPM program. An objective of this study was to determine differences, if any, in HWA populations between sampling years and among hemlock sites, years post-treatment, and hemlock DBH size classes. This study also assessed if the health of imidacloprid-treated hemlocks, as determined by canopy health characteristics, was affected by observed HWA populations.

Materials and Methods

Site and Hemlock Selection. Information from the GRSM hemlock treatment database was used to select potential study sites. Discussions with park personnel and site visits further narrowed appropriate sites to those of similar elevation and forest composition, where a single imidacloprid basal drench treatment was applied to hemlocks 4 – 7 yr prior to the initiation of sampling. Sites in close proximity, which were treated before a drought during the summer of 2007, were selected to prioritize sites that experienced similar environmental conditions in the years since treatment. Because HWA management for many sites involved two or three

imidacloprid applications over time, availability of appropriate sites in GRSM where imidacloprid was applied only once was limited.

Eastern hemlocks (n = 103 in 2012 and n = 102 in 2013) were selected from three sites. Anthony Creek (35° 35.682 N, 83° 45.845 W; 21 ha; elevation: 671 m) and Hesse Creek (35° 40.190 N, 83° 52.126 W; 6 ha; elevation: 311 m) are located on the western side of GRSM. Mountain Homes, Inc. (35° 40.574 N, 83° 52.144 W; 17 ha; elevation: 350 m), a private landowner community, is located on land adjacent to the western boundary of the park. Site locations and treatment histories are described in Benton et al. (2015). Hemlocks at each site ranged from 18.3 to 36.6 m tall and were separated into four different DBH size classes, centered (± 2.5 cm) around 30.4, 45.7, 61.0, 76.2 cm DBH (henceforth referred to as 30, 45, 61, and 76 cm).

Imidacloprid Application. Basal drench imidacloprid treatments were applied once to all selected hemlocks at each site. This application method involves pouring an aqueous suspension of a wettable powder formulation onto the surface soil within 0.6 m of the hemlock trunk. Some variation in imidacloprid dosage occurred among sites and hemlock size classes, which was unavoidable given the few appropriate sites available (due to multiple applications applied to other trees). Lower dose imidacloprid treatments (0.7 g active ingredient [AI]/2.5 cm DBH) were applied to all hemlocks at Anthony Creek and to hemlocks less than 63.5 cm at both Hesse Creek and Mountain Homes. Higher dose imidacloprid treatments (1.4 g AI/2.5 cm DBH) were applied to hemlocks 63.5 cm and larger at Hesse Creek and Mountain Homes, as specified by the HWA management plan for both locations.

Hemlocks at Mountain Homes (*n* = 33 trees) received imidacloprid treatments in 2007 (4 yr before the initiation of sampling in 2012). Hesse Creek (*n* = 35 trees) treatments occurred in late-2006 (5 yr before the initiation of sampling). Hemlocks at Anthony Creek (*n* = 35 trees [2012], *n* = 34 trees [2013]) were treated in mid-2006 (6 yr before sampling began) (see Benton et al. [2015] for further treatment timing description). Hemlocks sampled in 2012 were 4 – 6 yr post-treatment, and the same hemlocks were sampled 5 – 7 yr post-treatment in 2013. As some hemlocks were 5 and 6 yr post-treatment in both 2012 and 2013, more trees were sampled 5 and 6 yr post-treatment as compared to 4 and 7 yr post-treatment.

Foliage Sampling and HWA Population Assessment. During the winters of 2012 and 2013, nine branchlet samples (0.5 m long) were collected from each selected hemlock to assess HWA sistens generation populations on imidacloprid-treated trees. Collecting samples throughout the entire canopy to gain an understanding of overall infestation levels has been advocated in response to observations of patchy HWA distribution throughout the canopy (Joseph et al 2011a). Thus, three branchlets were randomly selected from each of three strata (lower third, middle third, and upper third) of the hemlock live canopy. Due to the height of the hemlock canopies and the remote locations of selected sites, tree climbers were employed to collect the branchlet samples. Depending on branchlet location in the canopy, pole pruners and hand pruners were used to detach branchlets. Once collected, samples were placed into pre-labeled plastic bags (7.5 L), which were then placed in cloth buckets (approximately 19 L) and lowered to the ground. Late instar or adult HWA on each branchlet were counted using 2.75× magnification goggles (Magni-focuser Model 107, Edroy Products Co., Inc., Nyack, NY) in the field, as time allowed, or in the laboratory. All branchlets collected from each hemlock were

transported to the laboratory in larger plastic bags (49 L). Upon arrival to the laboratory, samples were stored in a walk-in cooler (4° C) for up to 5 d to prevent mold growth until HWA could be counted on all of the branchlets. Counts from all nine samples were averaged to obtain a single measure for whole trees, when needed.

Canopy Health Assessments. The canopy health of each hemlock was assessed using the United States Forest Service's Crown Condition Classification (USDA Forest Service 2007, USDA Forest Service 2011) during early spring of both 2012 and 2013. Canopy assessments occurred before leaf-out of deciduous tree species to ensure better visibility of hemlock canopies. Live crown ratio, crown density, foliage transparency, and crown dieback were determined for each hemlock. Each canopy health characteristic was measured in percentages (0-100%). Canopy health characteristics measured were assessed to determine if they were associated with HWA population levels.

Data Analysis. All data were entered in an Excel file (Microsoft, Redmond, WA) and were analyzed using SAS (SAS Institute 2008). As the objectives of this study were to assess overall HWA populations and hemlock health, the entire hemlock was considered the experimental unit. HWA populations on each hemlock were assessed using a zero-inflated negative binomial model using SAS Genmod procedure to determine differences between sampling years (2012 and 2013) and among sampling sites (Anthony Creek, Hesse Creek, and Mountain Homes), years post-treatment (4, 5, 6, 7), and hemlock size classes (30, 45, 61, and 76 cm DBH). A zero-inflated negative binomial model is based on the concept that excess zeros in a dataset can be generated by a separate process than non-zero count data, thus the zero values and count data are modeled independently as a zero-inflated model and a count model, respectively (UCLA 2011). In this

approach, zero data (i.e., HWA absence) can be expected when imidacloprid or imidacloprid metabolite concentrations are high enough to cause HWA suppression, while HWA presence is expected when pesticide concentrations are too low to reduce populations. The zero-inflated model (hereafter referred to as "zero model") analyzed differences among hemlocks with no HWA. The count model analyzed data using mean HWA populations from hemlocks (of the mean levels of HWA per branch on the nine collected branchlets) using only data from hemlocks where HWA was present, thus hemlocks with no HWA were excluded from the count model. For example, if only 25% of hemlocks had HWA present in 2012, then the count model would be based on data from only these hemlocks, and data from the other 75% of hemlocks with no HWA present would not be included in the means. Zero model estimates and count model means generated by the zero-inflated binomial model for yr post-treatment and hemlock size classes were analyzed by either simple linear regression or polynomial regression to determine overall trends in the HWA population over time and as hemlock DBH increased ($P < 0.05$). Zero model estimates were subtracted from 1.0 to display data as percent of hemlocks with HWA for graphical comparison with count model means.

Canopy health characteristics (i.e., live crown ratio, crown density, foliage transparency, and crown dieback) were compared to HWA populations using simple linear regression and a mixed model ANOVA, with least significant difference (LSD) for mean separation, using a *P* value of < 0.1 rather than < 0.05 for significance as the method of canopy health ratings is somewhat subjective. Normality of canopy health characteristics was assessed using Shapiro-Wilks and Kolmogorov-Smirnov tests. When necessary, canopy data were log transformed (ln[x + 0.1]) to improve normality for mixed model ANOVA analyses. The regression analysis

compared canopy health characteristics to the average number of HWA per branchlet on each hemlock. The mixed model ANOVA analyzed canopy health characteristics among hemlocks with various levels of infestation, measured by total number of branchlets per hemlock with HWA present. Random effects in the mixed model ANOVA were year, site, yr post-treatment, and imidacloprid dosage level. Canopy health data were not available for four hemlocks due to complications with obtaining data in the field.

Results and Discussion

HWA Population. Applications of 0.7 and 1.4 g AI/2.5 cm DBH imidacloprid treatments in mixed DBH size hemlock stands resulted in effective HWA suppression 4 – 7 yr post-treatment. HWA were not present on 92.6% of the 1,845 branchlets collected, thus HWA were present on only 137 branchlets. The number of HWA per branchlet ranged from 1 – 1,604, with less than 100 HWA observed on 62.3% of the 137 branchlets where HWA was present. No HWA were present on any of the nine collected branchlets from 68.3% (n = 140) of the sampled hemlocks (n = 205) (Figure 13). Seven hemlocks (3.4%) had an average HWA per branchlet in excess of 100 HWA, while 26 (12.7%) and 32 (15.6%) hemlocks had > 0 – 10 and > 10 – 100 HWA per branchlet, respectively.

Management programs for HWA IPM can have numerous objectives, such as suppression, biological control, and simultaneous use of both chemical and biological control tactics. It is important to determine where the HWA IPM management plan used in this study fits in the scheme of desired IPM program outcomes. HWA suppression numerous years post-treatment using similar (approximately 0.75 – 1.5 g AI/2.5 cm DBH) and lower (0.15 and 0.35 g

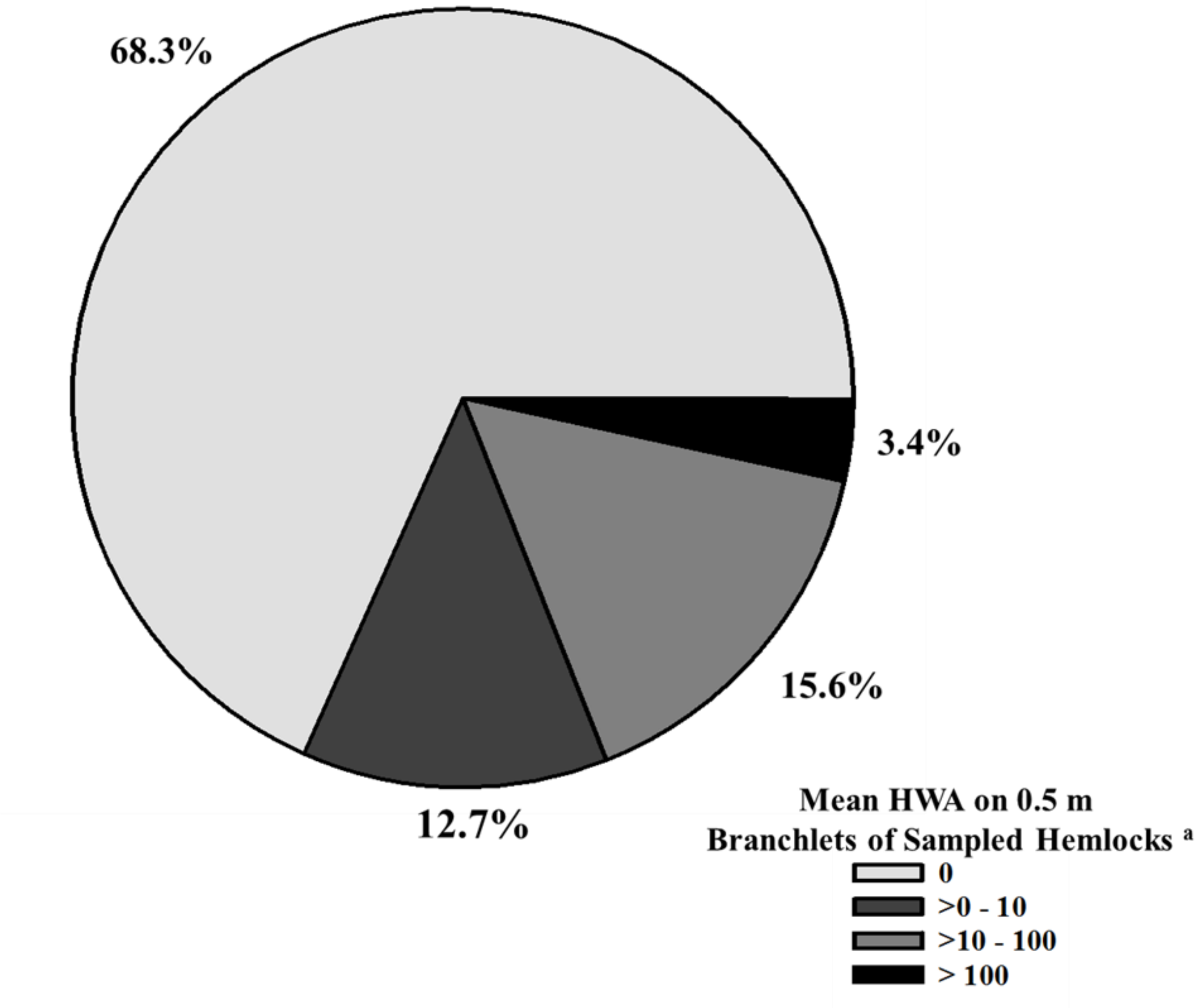


Figure 13. Percentages of mean hemlock woolly adelgid population ranges on sampled hemlocks (n = 205).

[1]mean HWA levels on nine sampled hemlock branchlets.

AI/2.5 cm DBH) dose imidacloprid treatments have been observed in other studies. However, HWA re-population levels observed in this study were lower than those observed when applying low-dose (0.15 and 0.35 g AI/2.5 cm DBH) imidacloprid treatments (Joseph et al. 2011b, Eisenback et al. 2014, Mayfield et al. 2015). Hemlock woolly adelgid densities > 1 HWA/cm foliage have been suggested for integrating chemical and biological control (Joseph et al. 2011b). As only 2% of branchlets in the current study had > 3 HWA/50 cm branchlet, observed levels of HWA may not be sufficient for integrating this treatment method with biological control, even 4 – 7 yr post-treatment. However, for IPM programs with the goal of maximizing the temporal effects of pesticide use, suppression was observed as long as 7 yr post-treatment at Anthony Creek using 0.7 g AI/2.5 cm DBH imidacloprid treatments.

In addition to low HWA population levels, patchy HWA distribution throughout the canopy was observed. Most often when HWA was observed on a sampled hemlock, only one of the nine collected branchlets from that particular hemlock contained HWA. Other studies have observed a similar patchy HWA distribution (Joseph et al. 2011a, Eisenback et al. 2014). The observed patchy distribution of HWA is likely related to the observed high variability of both imidacloprid and olefin translocated to foliage in the sampled hemlocks (Benton et al. 2015). Such patchiness may increase the opportunities for predators to sustain themselves within forest hemlocks previously treated with imidacloprid.

Year. Hemlocks with no HWA on sampled branchlets declined significantly from 77.0% in 2012 to 55.5% in 2013 (P = 0.031), meaning that the number of hemlocks with HWA was greater in 2013 compared to 2012 (Table 11, zero-inflated model). When HWA was present on hemlocks, mean HWA populations were significantly higher in 2013 compared to 2012 (P =

Table 11. Zero-inflated negative binomial model analysis of the effects of year, site, years post-treatment, and size class on observed hemlock woolly adelgid populations.

Category	Variables	Zero-Inflated Model		Count Model	
		Means[1]	Confidence Limits	Means[2]	Confidence Limits
Year	2012	77.0a[1,3]	60.9-87.9	61.8b[3]	21.2-172.6
	2013	55.5b	36.1-73.3	256.1a	121.0-542.0
Site	Mountain Homes	73.9b	58.6-85.0	181.4a	59.5-552.7
	Hesse Creek	84.3a	66.5-93.6	28.2b	6.2-128.9
	Anthony Creek	36.0b	16.3-61.9	389.9a	196.1-775.0
Years Post-Treatment	4	75.0ab	53.8-88.6	116.1a	27.8-484.4
	5	91.8a	67.9-98.3	5.4b	0.49-59.5
	6	63.0bc	45.6-77.6	234.5a	97.9-561.3
	7	46.4bc	24.5-69.8	338.7a	151.7-756.0
Size Class[4]	30	46.6b	22.3-72.7	94.3a	38.9-228.3
	45	65.7ab	45.1-81.7	254.5a	101.1-640.4
	61	71.1ab	53.3-84.1	88.8a	27.7-284.4
	76	81.0a	55.2-93.6	117.7a	20.3-680.9

[1]The Zero-inflated model uses the percentage of hemlocks with no HWA. Means are the percentage of hemlocks that had no detection of HWA.
[2]Means in the count model are the means only from hemlocks when HWA was present. Excess zero data from hemlocks with no HWA are not included in the means.
[3]Means within a column and category followed by the same letters are not significantly different ($P < 0.05$).
[4]cm diameter at breast height (DBH).

0.010) (Table 11, count model). So, not only did presence of HWA positive trees increase, but HWA abundance on branches with positive detections increased as well.

Observed increases in HWA populations occurred between 2012 and 2013 simultaneously with significant decreases in both imidacloprid and olefin concentrations in the foliage of the same sampled hemlocks occurred between 2012 and 2013 (Benton et al. 2015). The LC_{50} of imidacloprid is 112 and 300 ppb for HWA, as determined by 15 and 20 d laboratory dose–response assays (Cowles et al. 2006, Coots 2012). The concentrations of imidacloprid in all hemlocks sampled in this study were below the LC_{50} for HWA (Benton et al. 2015). However, in 2012 the mean concentrations of olefin were above the 6 ppb LC_{50} for HWA, as determined by 15 d laboratory dose-response studies (Coots 2012). Olefin concentrations were below the LC_{50} in 2013, which may explain the increase in HWA populations between 2012 and 2013. While the concentrations of both imidacloprid and olefin were below the LC_{50} in 2013, 55.5% of hemlocks had no HWA on sampled branchlets.

Site. The percentage of hemlocks with no HWA on sampled branchlets at each site ranged from 36.0 – 84.3%. Hesse Creek had significantly more hemlocks with no HWA compared to Anthony Creek ($P < 0.001$) and Mountain Homes ($P = 0.005$). There was no significant difference in the percentage of hemlocks for which HWA was not detected at Anthony Creek and Mountain Homes ($P > 0.05$) (Table 11, zero-inflated model). Mean HWA populations on hemlocks where HWA was present were significantly lower at Hesse Creek (28.2 HWA per branchlet) than at either of the other two sites, Mountain Homes (181 HWA per branchlet; $P <$ 0.037), and at Anthony Creek (389.9 HWA per branchlet; $P < 0.002$). No difference in mean HWA populations was detected between Mountain Homes and Anthony Creek ($P > 0.05$) (Table

11, count model). Hesse Creek had both fewer trees with positive detection from samples and lower HWA populations compared to the other sites.

Mean concentrations of olefin were above the 15 d LC_{50} at all sites in 2012 and below the 15 d LC_{50} at all sites in 2013 (Benton et al. 2015). The observed differences in infestation levels between Hesse Creek and Anthony Creek sites are not accounted for by differences in insecticide residues. There was no difference in olefin concentrations between these sites (Benton et al. 2015). The observed differences in HWA populations between sites could be caused by localized site-specific HWA densities in the surrounding landscape, differences in the proportion of hemlocks relative to hardwoods, tree condition at the time of treatment, or other variables that we did not investigate. It is important to note that although imidacloprid and olefin concentrations were below the LC_{50} at each site, HWA populations at all sites were suppressed.

Years Post-Treatment. The zero-inflated analysis investigated presence/absence data from whole trees. The percentage of hemlock trees on which HWA were absent from sampled branchlets decreased from 91.8% observed 5 yr post-treatment to 46.4% observed 7 yr post-treatment (Table 11, zero-inflated model). The analysis revealed a pattern of HWA infestation consistent with a decline in populations up to 5 yr, followed by a gradual increase (polynomial regression, $P = 0.044$, $R^2 = 0.87$) (Figure 14A). Statistically different groups of years were: year 5 from years 6 and 7 ($P = 0.032$ and 0.006, respectively), and year 4 was marginally different from Year 7 ($P = 0.049$).

The average counts of HWA populations from infested branches were only 5.4 HWA per branchlet when HWA was present 5 yr post treatment, which was significantly lower than 4, 6,

Figure 14. Linear relationships of the influence of years post-treatment and hemlock DBH size class on zero-inflated HWA population estimates (A and C) and adelgid population counts (B and D).

The influence of years post-treatment on percent of hemlocks with HWA (A) and mean HWA on each branchlet of each hemlock (B) was analyzed with polynomial regression ($P < 0.05$). The influence of hemlock DBH size class on percent of hemlocks with HWA (C) and mean HWA on each branchlet of each hemlock (D) was analyzed by simple linear regression ($P < 0.05$). Regression lines are not presented when no significant relationship was found.
Significant differences as determined by the zero-inflated binomial model (Table 2) are indicated by different letters in Figure 2A and C when significant linear relationships were documented.

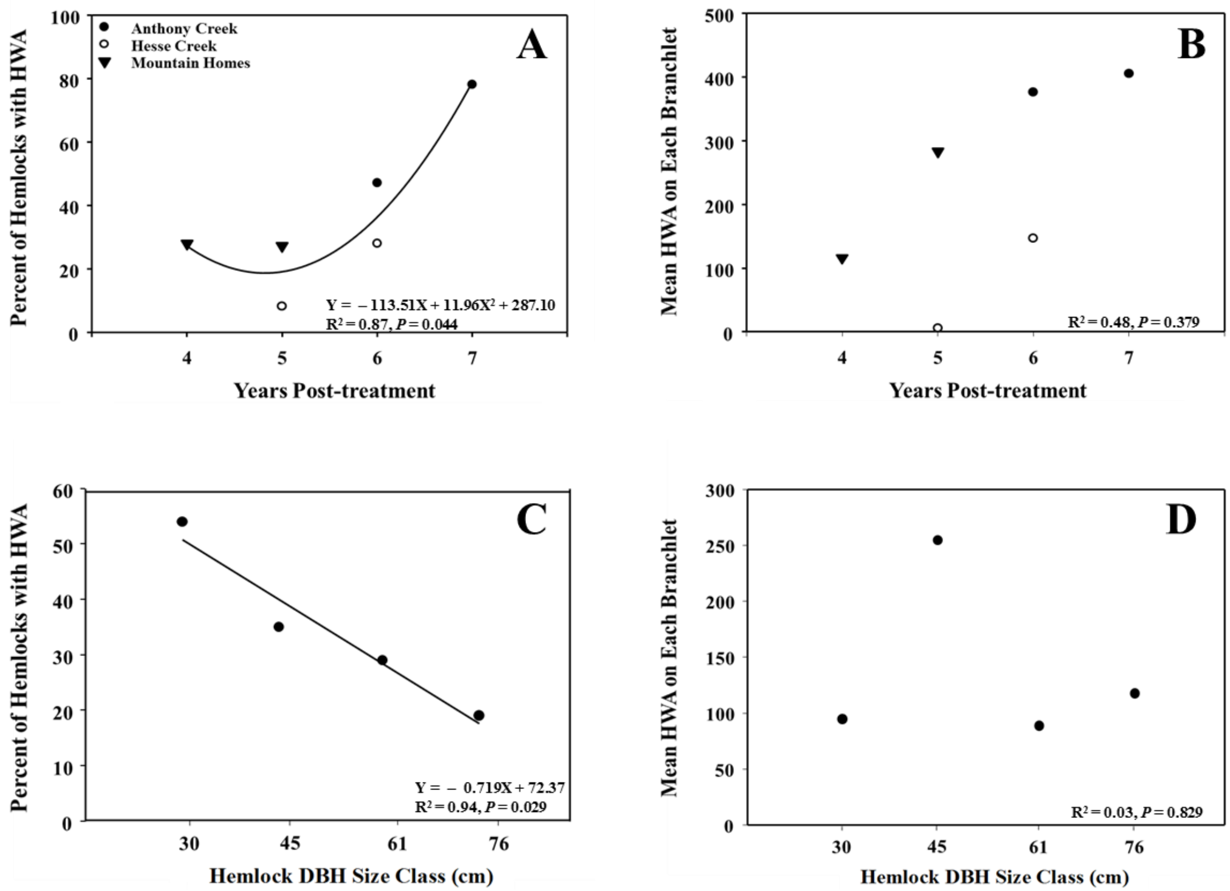


Figure 14. Continued

and 7 yr post treatment (*P* = 0.025, 0.002, and 0.001, respectively) (Table 11, count model). No difference in mean HWA populations was detected between 4 and both 6 and 7 yr post treatment (*P* > 0.05). While infested hemlocks 6 and 7 yr post-treatment had mean HWA populations of 234.5 and 338.7 HWA per branchlet, respectively, these populations did not significantly differ (*P* > 0.05). No polynomial relationship was observed between mean HWA populations and yr post-treatment for these count data (*P* = 0.379) (Figure 14B). Overall the zero model provides a clearer assessment of HWA population relationships than the count model, due to high variability in count data, as indicated by 95% confidence limits (Table 11). When pesticide levels in individual branchlets are insufficient to cause population suppression, the situation lends itself to highly variable HWA counts on each branchlet, which makes relationships analyzed by the count model more difficult to interpret. Thus, highly variable HWA populations observed in this study are more suited for analysis by a zero model (i.e., presence/absence data for trees).

The highest percentage of hemlocks with no HWA present (91.8%) and the lowest mean population of HWA were observed 5 yr post-treatment. Thus, the percentage of trees with HWA was still decreasing at 4 yr post-treatment and was lowest 5 yr post-treatment, suggesting that maximum efficacy is observed a considerable time after treatment, likely due to chronic physiological effects from low concentrations of insecticide present in hemlock tissues. However, HWA populations start to recover at 6 and 7 yr post-treatment, which is consistent with the observation that olefin concentrations decreased as yr post-treatment increased through this time period (Benton et al. 2015). Olefin concentrations only exceeded the 15 d LC_{50} 4 yr post-treatment (Benton et al. 2015). Concentrations of both olefin and imidacloprid were below

the LC_{50} 5 – 7 yr post-treatment. Even though insecticide concentrations were still below the LC50 threshold, it appears that imidacloprid and olefin continued to suppress HWA populations.

Size Class. For the zero-inflated analysis, the percentage of trees without HWA detection among hemlock size classes ranged from 46.6 – 81.0% of sampled trees (Table 11, zero-inflated model). The only significant difference in HWA absence among hemlock DBH size classes was detected between 30 and 76 cm hemlocks (P = 0.045). Significantly more hemlocks with no HWA occurred in the 76 cm size class compared to the 30 cm size class. HWA absence was similar between all other hemlock size class comparisons (P > 0.05). A significant negative linear relationship exists where the percent of hemlocks with HWA decreases as size class increases (P = 0.029, R^2 = 0.94) (Figure 14C).

For the count data, when HWA were present on hemlocks, mean HWA populations were between 88.8 and 254.5 HWA per branchlet (Table 11, count model). However, no significant differences were detected among any of the size classes (P > 0.05), and a significant linear relationship was not observed (P = 0.379) (Figure 14D). The HWA count mean for 45 cm hemlocks was 254.5 HWA per branchlet (Table 11). This HWA population level can be explained by previous work, because the 45 cm DBH size class was the one size class where models derived from residue data suggest that dosage would have to be increased to reach equivalent levels imidacloprid and olefin compared to other size classes, as this is the upper-end DBH of the low treatment application (Benton et al. 2016a).

A trend of increases in the percentage of hemlocks with no HWA present as hemlock DBH increased was observed. The trend of increased HWA repopulation on smaller trees is supported by decreasing olefin concentration in smaller trees, which in turn may have been

driven by the use of a higher dose (1.4 g AI/2.5 cm DBH) for larger trees. Concentrations of olefin were above the 15 d LC_{50} in 61 and 76 cm hemlocks and below the 15 d LC_{50} in the 30 and 45 cm hemlocks (Benton et al. 2016a). It should be noted that the hemlocks sampled in Benton et al. (2016a) included most, but not all of the hemlocks sampled in the current study. However, 46.6 and 65.7% of hemlocks in the 30 and 45 cm size classes, respectively, had no HWA present while imidacloprid and olefin concentrations were not sufficiently greater than published LC_{50} values, to cause population suppression. Continued absence of HWA on some trees with low residues may also indicate the length of time for trees to become reinfested, when the HWA population has been suppressed among neighboring trees.

Suppression of HWA in the presence of concentrations of imidacloprid and olefin below the LC_{50} for HWA may be occurring due to three possible scenarios, individually or in combination: 1) the LC_{50} for the HWA sistens generation may be lower than average reported concentrations, 2) an additive effect may occur between imidacloprid and olefin, or 3) a cumulative effect of HWA suppression may occur over numerous generations. The first possible scenario is that the HWA sistens generation in field conditions may have a lower LC_{50} than laboratory toxicity assessments. Toxicity of a substance is determined by the dosage of the substance and the duration of exposure (Rondeau et al. 2014). Laboratory dose-response bioassays for HWA were conducted over the course of 15 – 20 d: by necessity these tests used the progrediens, which develop quickly (Cowles et al. 2006, Coots 2012). However, the HWA sistens generation is exposed to imidacloprid and olefin in excess of 9 mo. This increased exposure time may result in a lower required concentration of pesticide for efficacy in HWA control (Eisenback et al. 2010).

Additive effects of low concentrations of imidacloprid and olefin are likely, as both insecticides affect the same target site. While individually neither imidacloprid nor olefin has seemingly sufficient concentrations in hemlock foliage for HWA mortality, the combined effects of these residues may be sufficiently toxic to cause HWA mortality.

Lastly, the observed suppression of HWA may be due to the continued presence of active concentrations at or below a measured LC_{50} value, which may have a significant effect when mortality is compounded over several generations. For example, the 80% reduction in HWA populations over the first year of exposure relative to the untreated check provides close to the expected 96% mortality when populations were measured after 2 yr (survival of 20% per yr over 2 yr results in overall survival of 4%) (Cowles et al. 2006). By the same logic, 30% annual mortality compounded over 4 yr would result in 76% fewer adelgids than in untreated trees. Elimination of HWA from single hemlocks or entire stands of treated hemlocks could also lead to longer periods of protection arising from the length of time for that hemlock or stand to become reinfested.

Hemlock Canopy Health Characteristics. Live crown ratios of these forest-grown hemlocks ranged from 25% in a larger DBH hemlock to 100%, with a mean of 58.5% live crown (Figure 15A). The regression analysis showed no significant effect of mean HWA per branchlet on the observed live crown ratio ($F = 0.14$; df = 1, 199; $P > 0.10$). In addition, mixed model ANOVA did not detect an effect of the number of collected branchlets with HWA present from each hemlock on the live crown ratio ($F = 0.18$; df = 6, 187; $P > 0.10$) (Table12).

Hemlock crown density ranged from 15 – 70%, with an average of 44.2% (Figure 15B). Mean HWA per branchlet of each hemlock did not vary with crown density ($F = 0.81$; df = 10,

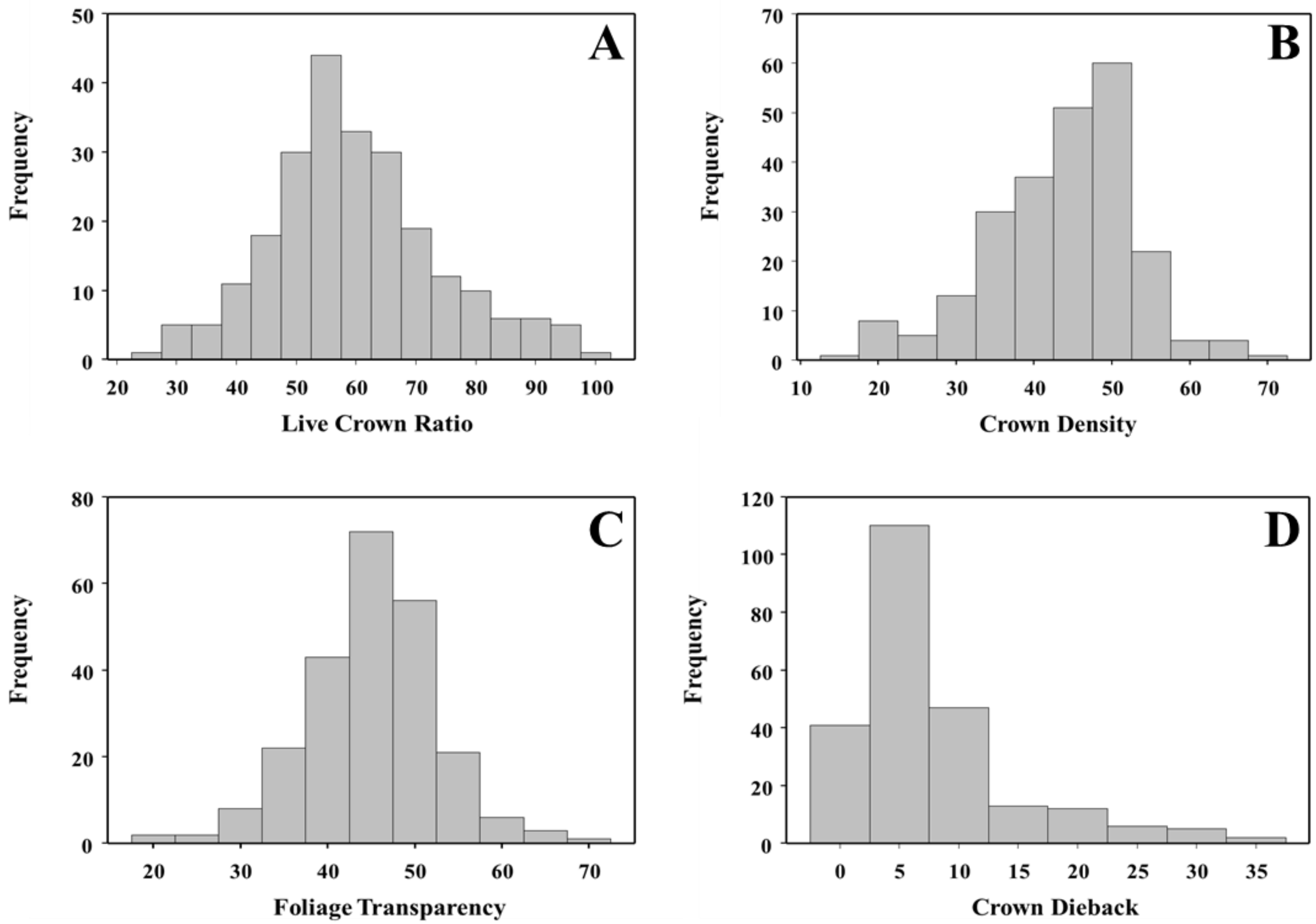


Figure 15. Frequency distributions of observed hemlock canopy health characteristics.

Table 12. Mixed model ANOVA comparison of the effects of the number of branchlets with hemlock woolly adelgid on hemlock canopy health characteristics.

Branchlets with HWA[1]	n[2]	Live Crown Ratio	Crown Density	Foliage Transparency	Crown Dieback
0	136	58.13 ± 3.06[3]	44.68 ± 2.93	44.76 ± 3.93	2.81 ± 2.81[4]
1	31	58.97 ± 3.78	45.61 ± 3.26	44.54 ± 4.09	2.83 ± 2.89
2	14	58.40 ± 4.73	45.39 ± 3.72	44.01 ± 4.31	6.45 ± 6.70
3	13	61.50 ± 4.86	48. 77 ± 3.78	45.72 ± 4.34	4.04 ± 4.26
4	4	58.32 ± 7.70	35.44 ± 5.28	51.94 ± 5.16	8.47 ± 10.01
6	2	64.57 ± 10.42	50.90 ± 6.85	41.92 ± 6.15	5.01 ± 6.84
8	1	62.07 ± 14.39	53.40 ± 9.18	36.92 ± 7.71	0.41 ± 0.82

[1]Number of the nine sampled branchlets on each hemlock in which HWA was present.
[2]Number of hemlocks within each group.
[3]Means ± standard errors.
[4]Means and standard errors displayed are back-transformed from log transformed means used in the statistical analysis.

199; $P > 0.10$). The number of branchlets with HWA present did not influence the observed crown densities ($F = 1.44$; df = 6, 187; $P > 0.10$).

Foliage transparency ratings ranged from 20 – 70%, and the average foliage transparency was 45.5% (Figure 15C). Foliage transparency was not affected by the mean HWA per branchlet ($F = 2.43$; df = 1, 199; $P > 0.10$), nor the number of collected branches with HWA present for each hemlock ($F = 1.13$; df = 6, 171; $P > 0.10$).

Hemlock canopies exhibited a 0 – 35% crown dieback of the outer branches of the canopy (Figure 15D). However, the average crown dieback was only 6.7%. Crown dieback data were log transformed (ln[x + 0.1]) to improve the normality for mixed model ANOVA analysis (Table 12). Dieback was not influenced by the mean HWA per branchlet ($F = 0.01$; df = 1, 199; $P > 0.10$). The number of collected branchlets with HWA present did not vary with crown dieback ($F = 1.59$; df = 6, 187; $P > 0.10$). No HWA were present on collected branchlets from 136 hemlocks in this analysis. Only two hemlocks had six infested branchlets, and one hemlock had eight infested branchlets. However, removal small number of high infestation data did not alter the results of any of the analyses (data not shown).

The lack of a causal relationship between HWA populations and hemlock canopy health is not unexpected, given the low HWA populations on sampled hemlocks. Only 3.4% of hemlocks had a mean HWA population greater than 100 HWA per 0.5 m branchlet. The highest mean HWA population on a hemlock in this study is 462.4 per branchlet, which is not excessively high given the densities HWA often attain in the field. In these hemlock populations containing low density HWA infestations, significant effects of HWA populations on hemlock

canopy health characteristics are not occurring. It appears that the densities observed in this study, HWA populations are not having detectable negative effects on hemlock canopy health.

A similar study assessed hemlock canopy health 2 yr after low-dose treatments (0.1 and 0.25 g AI/2.5 cm DBH) were applied. At 2 yr post-treatment hemlocks given low-dose treatments were found to have sufficient canopy health and HWA populations to support the combination of chemical and biological control tactics (Joseph et al. 2011b). Canopies of the same hemlocks were assessed at 6 and 7 yr post-treatment (Albert E. Mayfield III, personal communication), and low-dose imidacloprid treatment integration with biological control was deemed successful at 5 – 7 yr post-treatment (Mayfield et al. 2015). Hemlock canopy health from this study was measured at 4 – 7 yr post-treatment. While hemlocks given 0.7 and 1.4 g AI/2.5 cm DBH treatments were still healthy, they were generally in poorer health than those observed by Joseph et al. (2011b) at 2 yr post-treatment when given low-dose treatments . However, canopy health was poorer for other low-dose hemlocks at 6 and 7 yr post-treatment (Albert E. Mayfield III, personal communication) compared to the hemlocks measured in this study. The intent of the current 0.7 and 1.4 g AI/2.5 cm DBH imidacloprid treatment applications was to achieve high levels of HWA suppression over numerous years. This successful management tactic resulted in low HWA populations 4 – 7 yr post-treatment. While the current strategy may not integrate well with management programs seeking to integrate biological and chemical control tactics, it does result in greater hemlock canopy health numerous years after treatment.

In conclusion, 0.7 and 1.4 g AI/2.5 cm DBH imidacloprid basal drench treatments in mixed DBH hemlock stands were effective in suppressing HWA populations 4 – 7 yr post-

treatment. While Hesse Creek exhibited significant site-specific differences, generally the occurrence of HWA increased as the time since imidacloprid treatment increased. The prevalence of HWA decreased in the largest compared to the smallest hemlock size class, which is consistent with the larger trees having received higher doses of imidacloprid. Observed patterns in HWA occurrence can be linked to observed concentrations of olefin (Benton et al. 2015, 2016a). HWA populations were suppressed, when both imidacloprid and olefin concentrations in hemlocks were below the LC_{50} for HWA (Benton et al. 2015, 2016a). This phenomenon suggests a combination of factors may lead to better suppression than predicted. Sistens may have a lower LC_{50} for imidacloprid and olefin in field conditions; the effect between imidacloprid and olefin residues may be additive; and a cumulative effect of HWA suppression may occur over numerous years. It has long been recognized that imidacloprid has slow-acting effects on suppressing HWA populations. For example, Cowles et al. (2006) observed approximately 80% HWA mortality 1 yr after treatment and 96% mortality 2 yr after treatment.

Conclusions

The length of time (possibly 5 yr) to reach peak effects on suppressing HWA in this study was unexpected. However, a delay in the initial uptake by the tree may have occurred due to drought at the time of the initial insecticide application in 2007. We hypothesize that the active ingredient may have been immobilized in the soil until sufficient moisture was available to allow absorption by the roots. However, imidacloprid uptake at every management site is likely to be influenced by site-specific suites of environmental factors. In any case, the significant second order polynomial fit is consistent with the pattern over time of increased suppression of HWA,

followed by gradual decline of insecticide efficacy and eventual later increase of HWA numbers. Hemlock canopy health was not affected by the low HWA populations. No relationship could be elucidated between observed HWA populations and live crown ratio, crown density, foliage transparency, and crown dieback.

HWA IPM program managers must consider HWA management tactics and desired outcomes. The management tactic outlined in this study resulted in high levels of HWA suppression 7 yr after a single imidacloprid treatment at the sampled sites. HWA populations should be monitored for continued suppression at managed sites. However, should populations remain suppressed, the option of applying imidacloprid treatments as much as 7 yr after initial treatment can offer benefits to management programs. Nontarget environmental risks would be reduced, as imidacloprid is introduced to the system less often, and the high chemical and labor costs associated with treating individual trees in a forest would be mitigated by the infrequency of treatment required to maintain tree health.

CHAPTER V. ASSESSMENT OF IMIDACLOPRID TREATMENTS FOR HEMLOCK WOOLLY ADELGID ON STREAM WATER QUALITY IN THE SOUTHERN APPALACHIANS

This chapter is revised based on a paper published by Elizabeth Benton, Jerome Grant, Tom Mueller, Jesse Webster, and Rebecca Nichols:

Benton, E. P., J.F. Grant, T. C. Mueller, R. J. Webster, and R. J. Nichols. 2016. Assessment of imidacloprid treatments for hemlock woolly adelgid on stream water quality in the southern Appalachians. For. Ecol. Manag. 360: 152-158.

My contributions to this paper include (1) reviewing pertinent literature, (2) designing and conducting experiments, (3) processing, analyzing, and interpreting data, and (4) the majority of the writing.

Abstract

Imidacloprid, a neonicotinoid pesticide, is commonly used in hemlock woolly adelgid (HWA), *Adelges tsugae* (Annand) (Hemiptera: Adelgidae), pest management programs to preserve hemlock resources. Great Smoky Mountains National Park (GRSM) has an extensive HWA integrated pest management (IPM) program, with more than 200,000 individual hemlocks in the park having received imidacloprid soil treatments. A retrospective study was conducted in cooperation with GRSM to assess whether imidacloprid and two of its insecticidal metabolites (5-hydroxy and olefin) are present in surface waters (i.e., streams) associated with HWA imidacloprid treatment areas.

Thirty stream locations were sampled in GRSM to assess the presence and concentration of imidacloprid, 5-hydroxy, and olefin. Water samples were collected from 10 streams downstream from riparian areas where hemlocks received imidacloprid soil treatments and immediately upstream from hemlock treatment areas in each of the selected 10 streams. In addition, water samples were collected from 10 control streams each in close proximity to one of the 10 streams flowing through treatment areas. The concentrations of imidacloprid, 5-hydroxy, and olefin in parts per trillion (ppt) were determined by liquid chromatography mass

spectrometry (LC/MS). Data analysis included historical treatment data from GRSM. Data were analyzed using a Kruskal-Wallis test ($P < 0.05$), least significant difference (LSD), and a multiple regression ($P < 0.05$).

Imidacloprid, in concentrations ranging from 28.5 to 379 ng L^{-1}, was detected in 7 of the 10 downstream sampling locations. Upstream or adjacent stream locations did not have detectable concentrations of imidacloprid. Five-hydroxy and olefin were not detected in any streams. A positive relationship between the total amount of imidacloprid applied to a hemlock treatment area and the concentration of detectable imidacloprid in the associated stream was observed. However, while imidacloprid was detected in streams associated with hemlock treatment areas, the concentrations are below USEPA chronic and acute aquatic life benchmarks for fish (1,200 and 41,500 µg L^{-1}, respectively) and aquatic macroinvertebrates (1.05 and 34.5 µg L^{-1} , respectively). Since the amount of imidacloprid applied in a treatment area has an influence on the concentration of imidacloprid in streams, resource managers must carefully consider the frequency and extent of imidacloprid applications to meet management goals while providing minimal environmental impact.

Introduction

Hemlock woolly adelgid (HWA), *Adelges tsugae* (Annand) (Hemiptera: Adelgidae), an invasive insect from southern Japan (Havill et al. 2006), was unintentionally introduced to the eastern United States in the 1950s (Stoetzel 2002). HWA feeds on eastern hemlock, *Tsuga canadensis* (L.) Carriére, a slow-growing species that inhabits a distinctive ecological niche and is an important component of many forest types (Orwig and Foster 1998, Ward et al. 2004). As

the dominant shade-tolerant conifer in its habitat, eastern hemlock plays a vital ecological role in southern Appalachian forests, and that role cannot be filled by any other native evergreen tree species (Orwig and Foster 1998, Ward et al. 2004). Many species depend on eastern hemlock and will be negatively impacted by its decline (Wallace and Hain 2000, Hakeem 2008, Dilling et al. 2007, 2009, Coots et al. 2012). Unfortunately, as eastern hemlock has exhibited no visible resistance against the adelgid (McClure 1995) and no native predators are capable of suppressing adelgid populations (McClure 1987), excessive mortality and decline has occurred throughout most of the natural range of this native tree species in the eastern United States (Lambdin et al. 2006).

Great Smoky Mountains National Park (GRSM) launched an aggressive integrated pest management program against HWA to reduce damage to its hemlock resources once HWA was documented in the Park in 2002. Horticultural oil sprays, biological control (i.e., predatory beetles), and systemic imidacloprid applications have been employed to suppress HWA populations. Imidacloprid, a neonicotinoid pesticide, is the primary management tactic used in this program in the Park, where it is applied in GRSM as soil injections within 30 cm of the hemlock trunk , basal drench (i.e., imidacloprid solution is poured on the soil within 30 cm of the hemlock trunk), stem injections, or as a dissolvable pellet (CoreTect). Over 200,000 trees, many in riparian areas, have received imidacloprid soil treatments (Jesse Webster, personal communication).

Imidacloprid has been used for pest control since the early 1990s (Diehr et al. 1991) and is applied in agricultural, forestry, and urban settings to suppress a variety of pest species (Jeschke et al. 2011, Goulson 2013). The chemical structure of imidacloprid is similar to

nicotine (Figure 16) (Matsuda et al. 2001), and it functions similarly by acting on nicotinic acetylcholine receptors in the central nervous system of insects (Nauen and Bretschneider 2002). Neonicotinoids are commonly used because they are selective for treating arthropod pests, have low fish and mammalian toxicity, and can be applied by various methods (Sánchez-Bayo and Hyne 2014). However, concerns about the potential negative impacts of imidacloprid to surface water quality, aquatic macroinvertebrates, pollinators, and other non-target organisms have been expressed (USEPA 2008b, Dilling et al. 2009, Pestana et al. 2009, Goulson 2013).

Because imidacloprid can be toxic to aquatic macroinvertebrates if the dosage is high enough (Alexander et al. 2007, Pestana et al. 2009), its ability to leach into surface water and persistence in aquatic systems are important. Movement of imidacloprid through the soil is a route of potential impact to surface water quality (USEPA 2008b). Similar to other pesticides, once in the environment, imidacloprid begins to degrade by biotic, abiotic, and photolytic degradation (Wamhoff and Schneider 1999), and some degradation products of imidacloprid, such as olefin, 5-hydroxy, 4-hydroxy, and dihydroxy, have insecticidal properties (Nauen et al. 1998, 1999). The persistence of imidacloprid and its metabolites in the environment will influence their potential to cause negative non-target impacts.

The persistence of imidacloprid in the soil, determined by its ability to bind to soil and its degradation in the soil column (Cox et al. 2004), can affect which compounds enter surface waters. The sorption of imidacloprid into soil is dependent on the concentration of imidacloprid and the organic matter content in the soil, as imidacloprid binds to organic matter

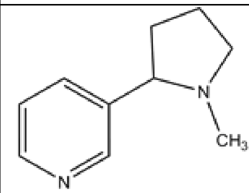
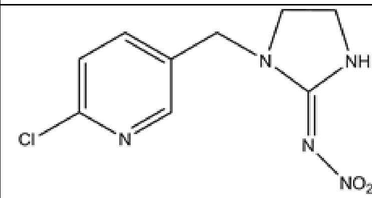
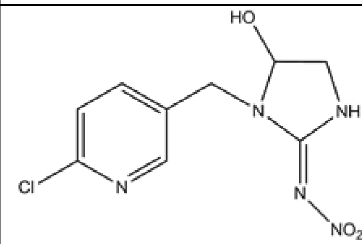
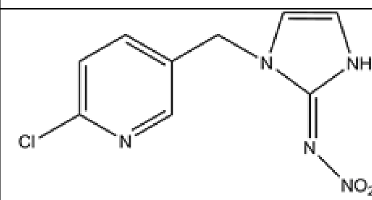
Common Name	IUPAC Name	Structure
Nicotine	3-(1-methyl-2-pyrrolidinyl)pyridine	
Imidacloprid	1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine	
5-hydroxy	1-(6-chloro-3-pyridylmethyl)-2-(nitroimino)imidazolidin-5-ol	
Olefin	1-(6-chloro-3-pyridylmethyl)-*N*-nitro-1,3-dihydro-imidazol-2-ylideneamine	

Figure 16. The UIPAC[1] names and chemical structures of nicotine, imidacloprid, and two insecticidal imidacloprid metabolites (5-hydroxy and olefin).

[1]IUPAC = International Union of Pure and Applied Chemistry

(Mullins and Christie 1995, Cox et al. 1998). In soils with high organic matter content, such as those in GRSM, less leaching is expected (Cox et al. 1998).

Once imidacloprid enters surface water its ability to persist may be limited because imidacloprid photodegrades in water (Moza et al. 1998, Wamhoff and Schneider 1999). The half-life of imidacloprid in water has been recorded from one hour to three days (Agüera et al. 1998, Moza et al. 1998, Wamhoff and Schneider 1999), and half-life can vary by season, ranging from estimates of 8.6 –52.8 hours (Lu et al. 2015). In the absence of light, imidacloprid is stable in water for more than 12 hours. However, when exposed to light complete degradation has been documented in less than five hours (Agüera et al. 1998).

Possible non-target effects of imidacloprid in eastern hemlock systems in both terrestrial and aquatic habitats have been investigated by numerous researchers (Hakeem 2008, Dilling et al. 2009, Churchel et al. 2011). Imidacloprid applied to hemlocks by soil injection can move laterally and horizontally through the soil (Knoepp et al. 2012). In numerous studies imidacloprid has been documented in surface waters associated with soil applications of imidacloprid in agricultural areas (Starner and Goh 2012, Hladik et al. 2014, Main et al. 2014). Imidacloprid and its metabolites may move into the water column through leaf degradation, since imidacloprid, olefin and 5-hydroxy have been detected in hemlock foliage tissue (Dilling et al. 2010, Coots et al. 2013). A similar scenario has been documented in the laboratory using ash leaves, where imidacloprid was found to enter the water column as leaves from treated ash trees degraded (Kreutzweiser et al. 2007). Given the presence of imidacloprid in surface waters via various routes, imidacloprid treatments for hemlock conservation may pose potential risks to surface water quality. The purpose of this study is to assess if imidacloprid, 5-hydroxy, and

olefin are present in surface waters in GRSM as a result of imidacloprid treatments for the suppression of HWA and if any treatment area and timing factors contribute to observed concentration of the insecticidal chemicals in water.

Materials and Methods

Ten streams flowing through hemlock-dominant or co-dominant forest types in treatment areas were selected for this study (Table 13). Ten locations, one in each stream, were selected 10-100 m downstream from a treatment area, hereafter referred to as downstream. As a control, ten locations, one in each stream, were selected 10-100 m upstream from the treatment areas, hereafter referred to as upstream. In addition, ten streams were selected in hardwood-dominant forest types, in the same watersheds as the streams in treatment areas, and are henceforth referred to as adjacent streams. No imidacloprid treatments were applied upstream from the adjacent stream locations; thus, these locations serve as an additional control. Water samples were collected from 30 stream locations (10 upstream, 10 downstream, and 10 adjacent stream) (Figure 17) in GRSM to assess the presence and concentration of imidacloprid and two of its metabolites (5-hydroxy and olefin) (Figure 16).

Treatment areas contained between 100 and 1,000 hemlocks that received imidacloprid treatments. Hemlocks in the riparian corridors of treatment areas were treated one to eight years before sampling and received between one and three treatment cycles, depending on the site (Table 13). A treatment cycle may refer to a time when most trees in a treatment area were treated or when the hectarage of a treatment area was expanded. Due to hemlock health in selected treatment areas and the expansion or contraction of the size of treatment areas, the

Table 13. Imidacloprid treatment histories for streams in treatment areas where imidacloprid was used for the management of hemlock woolly adelgid, Great Smoky Mountains National Park.

Stream	First Treatment	Last Treatment	Sampling Date	Treated Hectares	Total kg.a.i.[1,2] Applied	kg.a.i. 1 yr Prior to Sampling[3]	Treated Stream Length (m)	Treatment Cycles
Alum Creek	9/2004	8/2011	6/2012	19.0	14.8	0.2	4008	5
Camel Hump Creek	N/A[4]	N/A	5/2012	N/A	N/A	1.2	353	N/A
Cane Creek	2/2005	10/2010	2/2013	14.5	6.3	0	4178	3
Chasteen Creek	1/2005	6/2009	12/2012	42.6	16.8	0	8766	4
Dunn Creek	4/2005	9/2010	6/2012	47.1	114.0	0	1046	6
Indian Camp Creek	5/2005	9/2010	6/2012	N/A	N/A	0	9899	N/A
Indian Creek	9/2005	6/2011	8/2012	47.2	38.3	0	5046	5
Kingfisher Creek	5/2004	10/2012	10/2012	29.4	20.9	9.7	1773	4
Panther Creek	8/2011	4/2012	8/2012	26.6	1.8	1.8	3811	1
Shop Creek	4/2011	6/2011	10/2012	23.3	7.6	0	2249	1

[1]Kilograms active ingredient.
[2]Total kg.a.i. applied in the treatment area.
[3]kg.a.i. applied in the treatment area one year before water samples were collected.
[4]All data were not available for Camel Hump Creek and Indian Camp Creek.

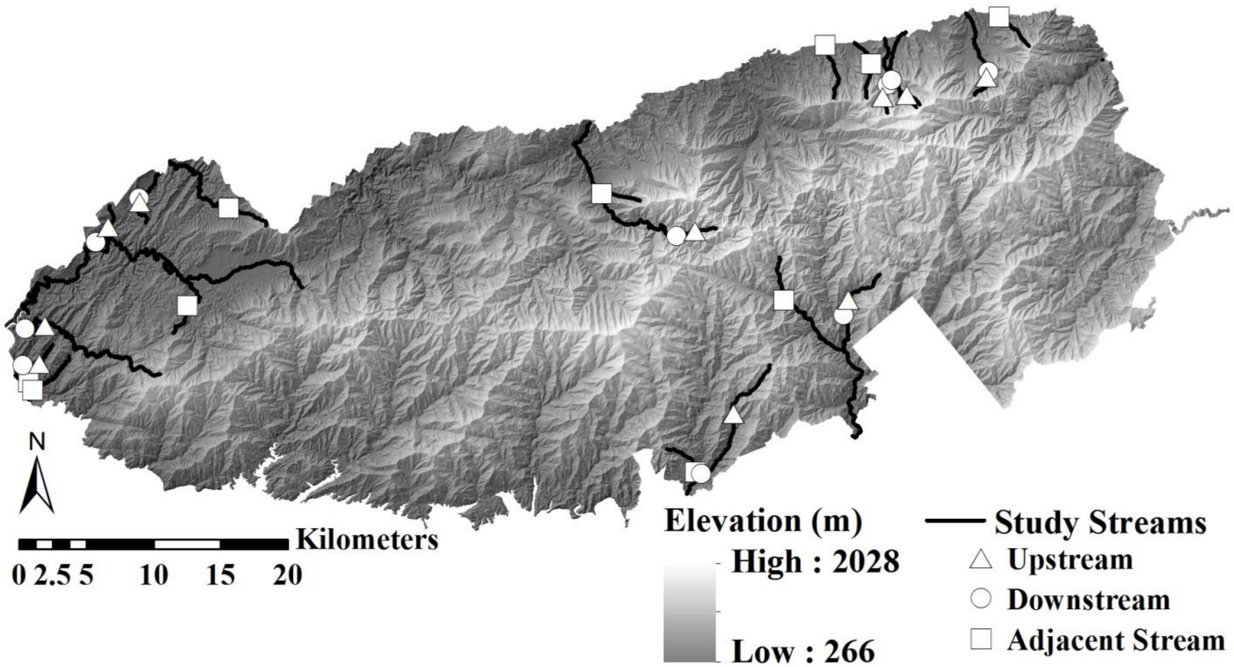


Figure 17. Stream sampling locations in the Great Smoky Mountains National Park.

number of trees per treatment area was not consistent among treatment cycles. For example, a larger treatment area may have had many treated hemlocks initially, but with hemlock mortality due to HWA in that area, fewer trees would have been treated during the next cycle. A few trees near campsites also may have had an initial treatment and later the larger area around the campsite was treated.

Imidacloprid was applied as a basal drench, which involves pouring a wettable powder solution of imidacloprid around the base of hemlock trees approximately 30 cm from the trunk. Trees smaller than 63.5 cm diameter at breast height (dbh) were treated with 0.7 g.a.i. (grams of active ingredient) per 2.5 cm dbh, and trees 63.5 cm dbh and larger were treated with 1.4 g.a.i. per 2.5 cm. Imidacloprid rates per hectare did not exceed the maximum allowable rate of treatment (0.4 kg per hectare per year) (Bayer 2006).

Samples were collected from each selected location (either upstream, downstream, or adjacent stream) during a single sampling event. During a sampling event three replicate water samples (1 L) were collected mid-channel and mid-depth from each stream sampling location using amber glass bottles (1 L) with Teflon lined lids. Glass bottles were placed into the water column, lid down. The bottle was then turned with the opening facing upstream to allow the bottle to fill with stream water. Containers were transported to and from the field in cooler bags (25 x 15 x 15 cm). Sampling locations were often in remote areas, so the cooler bags were placed in large backpacks for transport to the laboratory, where samples were stored in a walk-in cooler at 4°C until processed. Samples were processed within 3 wk of the collection date. All samples were collected between May 2012 and January 2013.

The amount of sample collected was sufficient to allow for concentration detection in parts per trillion (ng L^{-1}). All methods were optimized for greater sensitivity to determine low levels of imidacloprid in the environmental samples using liquid chromatography mass spectrometry (LC/MS). Sample preparation prior to analysis utilized an Empore aqueous extraction system (Mueller et al. 2000, Mersie et al. 2002, Senseman et al. 2003). This procedure passes the water sample through a 17 mm C 18 embedded filter allowing the matrix to pass through unimpeded and capturing the analytes of interest, imidacloprid, 5-hydroxy, and olefin. Preliminary studies examined the recovery of fortified imidacloprid samples using our methodology, and indicated that recovery was 49.0 to 52.5% (data not shown). Repeated attempts to increase recovery trying a range of different solvents and operating parameters were not successful. While recovery in the import system of 49.0 to 52.5% was not ideal, the consistency and relative goodness of that 50% across several validation runs encouraged the use of the described procedures. In addition, the Empore solid phase extraction platform is widely recognized as an appropriate sample processing and concentrating procedure. Thus, the determination of concentration in our samples, recovery was determined to be 50%. The entire water sample (1 L) was passed through an Empore disk (3M) on an Empore 6 station extraction manifold and processed using standard laboratory procedures to prepare a given water sample for LC/MS analysis. First, the Empore disk was conditioned using methanol. Once the disk was conditioned, the water sample was added to a reservoir, which holds the water above the disk. Water was then drawn through the disk using a vacuum pump (GAST model P104 oil-less pump) operated at zero to seven bar of negative pressure. Residual water was removed from the disk using the vacuum pump to dry the disk. The sample was eluted using 10 mL of methanol

and collected in a 12 mL vial. This sample preparation resulted in a highly concentrated sample that was prepared for LC/MS. Processed samples were stored at 4°C until LC/MS analysis.

Chromatographic conditions included use of the C 18 column (Phenomenex, Inc) and isocratic mobile phase of 30% acetonitrile and 70% water (both with 0.1% formic acid to maintain constant ionic strength). Mass spectrometry conditions included drying gas flow of 5.0 L, nebulizer pressure at 4.14 BAR, drying gas temperature of 300°C, vapor temperature 250°C, capillary voltage 2,000 V, Corona current was set at 1.0, charging voltage was set at 2000, and the fragmentor setting was 70. The ionization hardware used was mixed mode-ESI-APCI. Apparent molecular mass units using the select ion monitoring mode determined the imidacloprid, 5-hydroxy, and olefin simultaneously. Approximate retention times for imidacloprid, 5-hydroxy, and olefin were 8.50, 5.98, and 5.26 minutes, respectively.
They were analyzed as a group and each run included individual standards for the parent and metabolites, with an external standard technique used. The conservative limit of detection (LOD) was 20 ng L^{-1}. Given the difficulty of sample collection and storage, the decision was made not to attempt to fortify deionized water samples in the field. Method development strongly indicated that procedures were robust and highly precise for the detection and quantification of the target compounds.

Rainfall data were obtained from the National Oceanic and Atmospheric Administration (NOAA) climate data website (NOAA 2015). NOAA weather stations closest to the watersheds of interest were used. Data three days prior to sample collection were used to determine how much rainfall had recently fallen in the sampled watersheds. Data were not used in the analyses

because rainfall records were not available for Camel Hump Creek and incomplete for Cane Creek and Chasteen Creek.

All data were stored using an Excel file (Microsoft, Redmond, WA). The three replicate samples collected at each sampling location were averaged, to obtain one concentration for each sampling location for use in data analyses. A Kruskal-Wallis Test was used to determine significant differences, if any, among ranks of concentration of imidacloprid found in upstream, downstream, and adjacent stream sampling locations ($P < 0.05$). The mean ranks were separated using least significant difference (LSD). A multiple regression analysis was used to determine if a relationship existed between treatment area information and time variables and the concentration of imidacloprid documented in streams in GRSM ($P < 0.05$). A backward elimination selection method was used to select the model that best explained the data. All data analyses were conducted using SAS (SAS Institute 2008). The Camel Hump Creek treatment area was never isolated as a separate site from a larger treatment area in regards to data entry, so accurate numbers on treatment time and site variables specifically to that smaller watershed are not available. Indian Camp Creek flows through numerous treatment areas, but does not have a distinct treatment drainage area for treatment time and site variables. Because all site data are not available for Camel Hump Creek and Indian Camp Creek they were not included in the multiple regression analysis.

Results

Imidacloprid was detected in streams associated with imidacloprid treatments for the control of HWA in this study (Table 14). All stream locations where imidacloprid was detected

Table 14. Concentration in ng/L (parts per trillion) of imidacloprid and downstream locations and rainfall totals three days prior to sample collection, Great Smoky Mountains National Park.

Stream Name	Imidacloprid Concentration	Rainfall (cm)
Alum Creek	28.5[1] ± 3.8[2]	2.44
Camel Hump Creek	<LOD[3]	Na[4]
Cane Creek	<LOD	0.53[5]
Chasteen Creek	36.8 ± 3.4	0[5]
Dunn Creek	379.1 ± 7.9	0.97
Indian Camp Creek	78.0 ± 8.0	0.97
Indian Creek	31.2 ± 1.5	3.35
Kingfisher Creek	33.6 ± 6.6	0
Panther Creek	<LOD	0.20
Shop Creek	82.2 ± 25.8	0.71

[1]Means are an average of the concentrations of the three samples collected at each sample location.
[2]Standard deviation
[3]Imidacloprid concentration was below the limit of detection (LOD) (20 ng/L).
[4]Rainfall data for Camel Creek were not available.
[5]Complete rainfall data were not available during the 3-d time period prior to sampling.

were downstream from imidacloprid treatment areas. Imidacloprid was detected in seven out of ten downstream locations, and imidacloprid concentrations ranged from 28.5 to 379.1 ng L^{-1}. In six of the streams where imidacloprid was detected the concentration of imidacloprid was below 100 ng L^{-1}. Dunn Creek, with a documented imidacloprid concentration of 379.1 ng L^{-1}, was the only stream where the concentration of imidacloprid was in excess of 100 ng L^{-1}. Three downstream locations, Camel Hump Creek, Cane Creek, and Panther Creek, had no samples that exceeded the LOD for imidacloprid. Samples from all upstream and adjacent stream locations did not exceed the LOD for imidacloprid (data not shown). All samples were below the LOD for 5-hydroxy and olefin (data not shown).

Rainfall amounts and imidacloprid concentrations detected in streams do not have a clear pattern. This may be, in part, due to the variety of treatment area conditions in the study. The two highest concentrations recorded, 379.1 and 78.0 ng L^{-1}, were detected in Dunn Creek and Indian Creek, respectively. Nearly 1 cm of rainfall occurred three days prior to sample collection, which may have influenced the observed concentrations. However, rainfall events in excess of 2 and 3 cm occurred before sample collection at Alum Creek and Indian Creek, respectively. While imidacloprid was detected in those streams, concentrations were only 28.5 and 31.2 ng L^{-1}. Little to no rain occurred prior to sampling at Panther Creek, Chasteen Creek, and Kingfisher Creek. Imidacloprid was detected at both Kingfisher Creek (33.6 ng L^{-1}) and Chasteen Creek (36.8 ng L^{-1}). Given the diversity of hectarage and imidacloprid usage in treatment areas, it would be difficult to perceive overall trends in imidacloprid concentrations in stream water based on rainfall.

A significant difference among upstream, downstream, and adjacent stream categories was detected (X^2 = 52.92, df = 2, P = < 0.001; Kruskall-Wallis). Downstream locations have a significantly higher mean rank for imidacloprid concentrations than upstream and adjacent stream locations (P < 0.05, LSD test). Locations downstream from imidacloprid treatment areas had significantly higher concentrations of imidacloprid than upstream and adjacent stream locations, both of which did not have detectable concentrations of imidacloprid.

The selected multiple regression model, which includes months since the first and last imidacloprid treatments, the number of treated hectares, and the total amount of imidacloprid applied to treatment areas, explains 97% of the variation in the data. The model overall was significant (P = 0.009), and all variables could explain at least 48% of the variation adjusted for the other variables. Given the adjustments made for the other variables in the model, the concentration of imidacloprid found in streams is positively related to the total amount of imidacloprid applied to treatment areas (Partial R^2 = 0.96, P = 0.002) (Table 15, Figure 18). Cane Creek and Panther Creek, two sites where imidacloprid was not detected, had the smallest amounts of imidacloprid applied to their treatment areas, 6.3 and 1.8 kg.a.i., respectively. Dunn Creek, which had an imidacloprid concentration of 379.1 $\mu g\ L^{-1}$, also received the greatest amount of imidacloprid applied to the treatment area (114.0 kg.a.i.). The concentration of imidacloprid detected at Dunn Creek is largely responsible for the slope of the relationship between the concentration of imidacloprid and the amount of imidacloprid applied to treatment areas. No significant relationship was detected between imidacloprid concentrations and treatment area variables when Dunn Creek was removed from the analysis and only lower concentration data points were considered (data not shown). However, data collected from Dunn

Table 15. Multiple regression associating imidacloprid concentration in streams with treatment area information and time variables.

Variable	DF	Parameter Estimate	Standard Error	t Value	Pr > |t|	Partial R^2
Intercept	1	99.3703	36.8975	2.69	0.074	-
Mo. Since First Treatment	1	-0.9091	0.3260	-2.79	0.069	0.7216
Mo. Since Last Treatment	1	1.4638	0.8674	1.69	0.190	0.4870
Hectares	1	-2.8644	1.1769	-2.43	0.093	0.6638
Total kg.a.i. Applied[1]	1	0.00402	0.0004	9.72	0.002	0.9694

[1]Total kg.a.i. applied in the treatment area

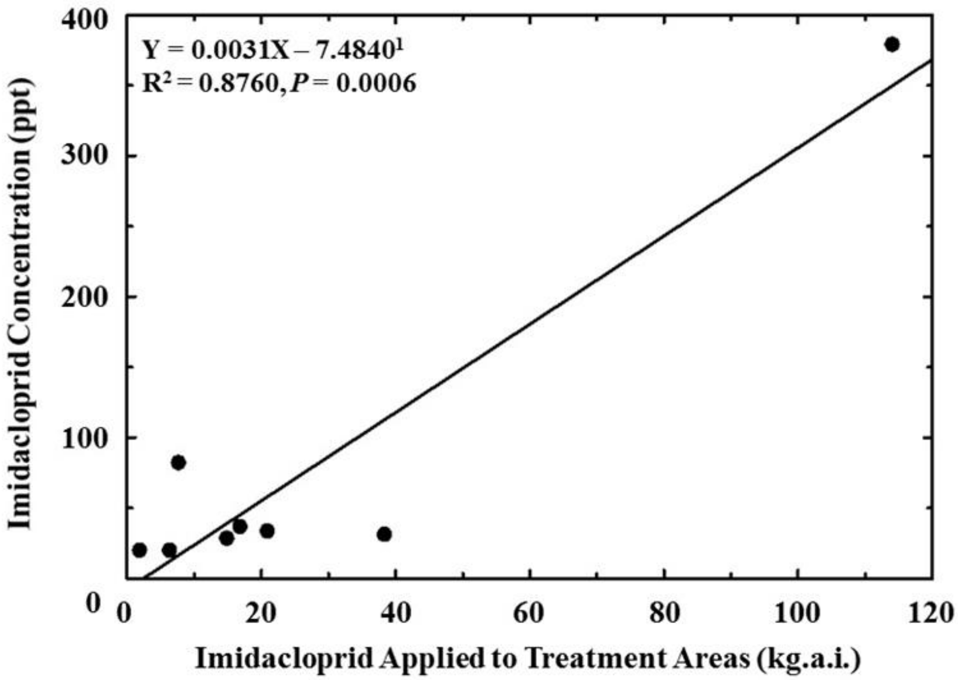


Figure 18. Relationship between the amount of imidacloprid applied to treatment areas and the concentration of imidacloprid observed in streams.

[1]No adjustments are made for other variables.

Creek is valid and explains much of the relationship between imidacloprid concentration in streams and the amount of imidacloprid applied in treatment areas (Table 15).

Discussion

The potential of imidacloprid from hemlock treatments to leach through soil, enter surface water, and cause associated negative impacts on water quality and aquatic biota is an issue that scientists, regulators, and land managers must consider. According to the USEPA, the Chronic and Acute Aquatic Life Benchmarks of imidacloprid for fish is 1,200 and 41,500 µg L^{-1}, respectively. Aquatic invertebrates have much lower Chronic and Acute Aquatic Life Benchmarks of 1.05 and 34.5 µg L^{-1}, respectively (USEPA 2008a). The LC_{50} (the concentration at which 50% of individuals of a taxa are killed) of imidacloprid for aquatic macroinvertebrates in 96 h exposure studies ranges from 0.65-12.94 µg L^{-1} (Alexander et al. 2007, Stoughton et al. 2008, Pestana et al. 2009). Sublethal effects on aquatic macroinvertebrates have been documented at concentrations of 0.10 to 3.00 µg L^{-1} in 96 h exposure trials (Azevedo-Pereira et al. 2011, Roessink et al. 2013). Sublethal effects of imidacloprid were observed in a mesocosm experiment using 12 µg L^{-1} imidacloprid pulses simulating rainfall event frequency and duration (Mohr et al. 2012).

Concern has been raised regarding the current method of using short-term exposure data to set water quality standards, as the cumulative effect of exposure to low imidacloprid concentrations is likely to have sublethal impacts on aquatic macroinvertebrates. In addition, the EPA Aquatic Life Benchmarks are higher than standards set by Canada, Europe, and the Netherlands (Morrissey et al. 2015). While negative effects of imidacloprid exposure on aquatic

macroinvertebrates have been documented, concentrations observed in this study are below concentrations documented to have negative acute and chronic effects. Six streams had documented imidacloprid concentrations that were less than 0.10 µg L^{-1}, which is one tenth of the USEPA Chronic Aquatic Life Benchmark. Dunn Creek was the only sampling location where imidacloprid concentration was in excess of 100 ng L^{-1}, and the observed concentration (379.1 ng L^{-1}) is below the above-mentioned USEPA Chronic and Acute Aquatic Life Benchmarks of imidacloprid for both aquatic macroinvertebrates and fish. In addition, preliminary results from a complementary study assessing aquatic macroinvertebrates in the upstream and downstream locations indicate similar abundance and taxa richness of environmentally sensitive aquatic macroinvertebrate taxa (unpublished data).

Imidacloprid has been previously documented in a stream associated with imidacloprid treatments for suppression of HWA. In that study, four streams were sampled for approximately two yr after imidacloprid soil applications, and only one sample, collected over 700 d after treatment, tested positive for imidacloprid. The concentration of imidacloprid in the only positive sample was < 1 µg L^{-1}. However the LOD for their study was 0.6 µg L^{-1} (Churchel et al. 2011), which is 30 times higher than the LOD in the current study. All documented concentrations of imidacloprid in our study were lower than the 0.6 µg L^{-1} LOD in Churchel et al. (2011). If methods used in that study had allowed for a lower LOD, then more positive samples may have been detected in streams associated with imidacloprid treatments for HWA. In addition to low documented presence of imidacloprid in streams, no negative effects on aquatic macroinvertebrates were observed in the stream where imidacloprid was documented (Churchel et al. 2011).

The absence of olefin and 5-hydroxy in stream samples was not unexpected. Olefin and 5-hydroxy are not the main metabolites of imidacloprid produced via photodegradation in water (Agüera et al. 1998, Moza et al. 1998, Redlich et al. 2007). However, since both metabolites are highly toxic insecticidal metabolites produced in numerous plant systems, including hemlock, it is important to establish the absence of olefin and 5-hydroxy in streams flowing through HWA treatment areas (Nauen et al. 1998, 1999, Coots et al. 2013).

Eastern hemlock is an important component of southern Appalachian riparian ecosystems with many aquatic and terrestrial species depending on its presence. With hemlocks in eastern forests declining, land managers must make difficult decisions involving positive and negative trade-offs of treatments for the protection of hemlock resources. Assessment of the presence and concentration of imidacloprid in streams as a result of imidacloprid treatments to hemlocks is an initial step to determine what negative consequences to surface water quality must be considered when making management decisions. Because the amount of imidacloprid applied in a treatment area has a significant effect on the concentration of imidacloprid observed in streams in this study, the frequency and extent of imidacloprid applications must be carefully considered. Land managers must decide if the risk of imidacloprid exposure to aquatic macroinvertebrates adjacent to areas of treated hemlock outweighs the benefits of preserving hemlock, which is a key species in many systems.

Conclusions

Imidacloprid was present downstream from imidacloprid treatment areas in seven of ten streams, and the presence of imidacloprid was not observed in upstream and adjacent stream

samples. The highest concentration observed, 379.1 ng L^{-1}, was below USEPA Aquatic Life Benchmarks for chronic toxicity of imidacloprid to aquatic invertebrates. Six of the seven streams where imidacloprid was documented had concentrations below 100 ng L^{-1}, less than one tenth of the USEPA Chronic Aquatic Life Benchmark. No obvious trends existed between the amount of rainfall prior to sampling and the observed concentration of imidacloprid in streams. A positive relationship between the total amount of imidacloprid that was applied in treatment areas and the imidacloprid concentration in streams was documented. Months since the first and last imidacloprid treatments as well as hectares treated explained at least 48% of the observed variation in imidacloprid concentration data. The insecticidal metabolites olefin and 5-hydroxy were not documented in any of the sampled streams. Knowledge about the presence and concentration of imidacloprid in multiple streams associated with HWA treatment areas can help land managers make calculated assessments of the risks and benefits of treating hemlocks with imidacloprid for the suppression of HWA. Based on these results, imidacloprid does appear in streams associated with HWA treatment areas. Concentrations detected are below USEPA Chronic and Acute Aquatic Life Benchmarks and should not negatively impact the aquatic community. Examination of the aquatic community composition among sites will be addressed in a separate paper.

CHAPTER VI. RISK ASSESSMENT OF IMIDACLOPRID USE IN FOREST SETTINGS ON THE AQUATIC MACROINVERTEBRATE COMMUNITY

Abstract

Imidacloprid is difficult to assess in natural settings due to the presence of numerous pollutants in many streams. Imidacloprid use for the suppression of hemlock woolly adelgid (HWA), *Adelges tsugae* (Annand) (Hemiptera: Adelgidae), in forests offers a rare opportunity to assess potential imidacloprid impacts to aquatic macroinvertebrates in pristine landscapes. Aquatic macroinvertebrate communities were assessed in nine streams in Great Smoky Mountains National Park (GRSM). The streams flow through hemlock conservation areas where imidacloprid soil treatments were applied for HWA suppression in riparian areas. Sites were located upstream (i.e., above imidacloprid treatment) and downstream (i.e., below imidacloprid treatment) from these conservation areas. Baseline species presence data were available from previous sample collections at downstream sites before imidacloprid treatments were initiated. Downstream and upstream sites did not vary in abundance, species richness, dominance, evenness, Shannon diversity, or mean tolerance values. Although comparisons of paired upstream and downstream sites showed differences in diversity in six streams, higher diversity was found more often in downstream sites. Functional feeding groups and life habits of macroinvertebrate communities did not vary between downstream and upstream sites. Downstream and baseline stream samples had no difference in overall richness, mean tolerance values, and life habits. Functional feeding group species richness varied, but variations did not indicate poorer quality downstream communities. Imidacloprid treatments applied according to USEPA federal restrictions did not result in negative effects to aquatic macroinvertebrate communities. These findings indicate that risks of imidacloprid use for HWA suppression to aquatic macroinvertebrate communities are low.

Introduction

Neonicotinoid pesticides are currently under much scrutiny due to concerns about non-target risks. Before the 1990s carbamates, pyrethroids, and organophosphates constituted the majority of insecticides used in agricultural systems. Neonicotinoids were developed in response to concern about the chronic and acute mammalian toxicity of these insecticide classes (Jeschke et al. 2011). Neonicotinoids, which irreversibly bind to nicotinic acetylcholine receptors (nACHRs) in post-synaptic nerve membranes in insects causing the eventual termination of nerve impulses, are the most widely used class of insecticides in the world (Nauen and Bretschneider 2002, Jeschke and Nauen 2005, Jeschke et al. 2011). Low toxicity to vertebrates occurs because neonicotinoids are less selective towards nerve receptors in vertebrates compared to insects (Tomizawa and Casida 2003). Thus, safety profiles for neonicotinoids to vertebrates are much better compared to other classes of pesticides.

The widespread use of neonicotinoids has a great potential to stray far from integrated pest management (IPM) tactics (Goulson 2013). Concern for non-target effects of neonicotinoids, especially imidacloprid, has increased. Factors of particular concern include environmental persistence, potential to leach into surface waters, toxicity to aquatic macroinvertebrates, and role in pollinator decline (USEPA 2008b, Pestana et al. 2009, Cresswell 2011, Starner and Goh 2012).

The movement of imidacloprid through the soil is a route of potential impact to surface water quality (USEPA 2008b). Imidacloprid photodegrades in water, where it has a half-life ranging from 1 h to 3 d (Agüera et al. 1998, Moza et al. 1998, Wamhoff and Schneider 1999). Because of this photodegradation timeframe, potential effects of aquatic macroinvertebrate

exposure to imidacloprid pollution in surface water are likely chronic rather than acute (Anderson et al. 2015). Chronic effects are sublethal effects after exposure to a lower concentration of a pollutant over a longer timeframe. Acute effects generally refer to lethal effects after a short-term, high concentration exposure to a pollutant. Chronic exposure to imidacloprid is expected to result in cumulative and usually permanent effects (Tennekes and Sánchez-Bayo 2011).

The United States Environmental Protection Agency (USEPA) lists imidacloprid as highly toxic to aquatic macroinvertebrates (USEPA 2008b). Caddisflies (Trichoptera), mayflies (Ephemeroptera), and true flies (Diptera) have been documented as the most sensitive taxa (Mohr et al. 2012). Aquatic macroinvertebrate toxicity data are most often generated from dose-response single species laboratory assays. These studies are commonly based on responses to short-term exposure times of 24 – 96 h. Lethal concentrations (LC_{50}) for true flies (*Simulium latigonium* Rubtsov [Diptera: Simuliidae]) and mayflies (*Epeorus longimanus* Eaton [Family Heptageniidae]) have been documented as low as 3.73 and 0.65 µg/L, respectively (Alexander et al. 2007, Beketov and Liess 2008).

A 28-d assessment was conducted to determine chronic effects of imidacloprid over longer timeframes to gauge likely impacts in natural systems (Roessink et al. 2013). Lethal concentrations (LC_{50}) were 0.195 and 0.316 µg/L for the mayflies *Cloeon dipterum* (L.) (Ephemeroptera: Baetidae) and *Caenis horaria* (L.) (Ephemeroptera: Caenidae), respectively. Immobilization (EC_{50}; effect concentration) was observed at 0.123 and 0.126 ppb, respectively (Roessink et al. 2013). Unfortunately, laboratory experiments consisting of single species analyses are not adequate to fully gauge potential ecological threats (Crane 1997).

Microcosm and mesocosm studies have been conducted to assess the effects of imidacloprid on macroinvertebrate communities in settings replicative of natural conditions (Mohr et al. 2012, Colombo et al. 2013). Aquatic macroinvertebrate communities were exposed to 12 µg/L imidacloprid pulses to simulate stormflow peaks in imidacloprid pollution. Negative long-term effects of imidacloprid pulses included reduction in abundance of chironomids (Diptera: Chironomidae), increased abundance of tolerant gastropods (Hygrophila: Limnaeidae), decreased emergence of adult mayflies (Ephemeroptera: Caenidae), less overall taxa emergence in the summer, and a decline in caddisfly nets (Trichoptera: Polycentropodidae) (Mohr et al. 2012, Colombo et al. 2013). However no difference was detected in the overall abundance of larval mayflies, caddisflies, and true flies (Mohr et al. 2012, Colombo et al. 2013).

To protect water quality from potential detrimental effects of imidacloprid pollution, many countries have set limits on imidacloprid concentrations in surface waters, with some researchers suggesting more stringent limits. Chronic and Acute Aquatic Life Benchmarks set by the USEPA are 1.05 and 34.5 µg/L, respectively (USEPA 2008a). Methods of USEPA limit determination are unclear. The limit in Canada is 0.23 µg/L for chronic exposure, which is based on the 28 d EC_{15} for reduced emergence of *Chironomus riparius* Meigen (Diptera: Chironomidae) of 2.25µg/L, divided by a safety factor of 10 (CCME 2007). Dutch standards for chronic and acute imidacloprid concentrations are 0.067 and 0.2 µg/L, respectively (RIVM 2008). Limit determination was based on the NOAEC (no observable adverse effect concentration) of 0.67 µg/L for *Chironomus tetans* F. (Diptera: Chironomidae). The NOAEC was divided by a safety factor of 10 and 3 for the chronic and acute limits, respectively (RIVM 2008).

A recent Dutch study recommended a 0.013 µg/L limit to protect sensitive aquatic taxa (Van Dijk et al. 2013). As imidacloprid co-occurred with other pesticides in many water samples used in limit determination, it has been suggested that linking imidacloprid alone to observed aquatic macroinvertebrate abundance was not appropriate (Vijver and van den Brink 2014). Another suggested chronic limit of 0.0083 µg/L was derived by using the 28 d EC_{10} (immobilization) of 0.024 µg/L for the mayfly *Caenis horaria* (Ephemeroptera: Caenidae) and dividing it by a safety factor of 3 (Smit et al. 2015).

Neonicotinoids, including imidacloprid, have been detected in surface waters in numerous studies. Concentrations of neonicotinoids documented in surface water samples reviewed from 29 studies showed average ambient concentrations of 0.13 ppb and average peak concentrations of 0.63 ppb (Morrissey et al. 2015). Sampled streams in the United States have concentrations of imidacloprid ranging from 0.05 – 0.67 µg/L (Hladik and Calhoun 2012, Starner and Goh 2012, Hladik and Kolpin 2015, Benton et al. 2016).

Imidacloprid is present in surface waters in concentrations that are expected to negatively affect aquatic macroinvertebrates. The presence of numerous pollutants in surface waters has prohibited assessing the isolated effects of imidacloprid in watersheds (Vijver and van den Brink 2014). However, the use of imidacloprid for suppression of hemlock woolly adelgid (HWA), *Adelges tsugae* (Annand) (Hemiptera: Adelgidae), offers a unique opportunity to assess imidacloprid in pristine landscapes.

Imidacloprid has been widely used for suppression of HWA, an invasive species threatening eastern hemlock, *Tsuga canadensis* (L.) Carrière, and Carolina hemlock, *Tsuga caroliniana* Engelmann (Pinales: Pinaceae), resources in forests in the eastern United States.

Eastern hemlock is a foundation species, providing many ecological services in forest settings (Orwig and Foster 1998, Ward et al. 2004). Hemlocks provide habitat for over 400 species of canopy arthropods and are a source of food and shelter for wildlife (Lapin 1994, Wallace and Hain 2000, Tingley et al. 2002, Dilling et al. 2007, 2009, Hakeem 2008, Mallis and Rieske 2011, Coots et al. 2012). Hemlock-dominated riparian areas have a marked effect on water temperature regimes and aquatic macroinvertebrate species composition (Snyder et al. 2002, Ross et al. 2003, Webster et al. 2012). The loss of this shade-tolerant conifer will have cascading ecological effects, as the role of hemlock cannot be filled by any other native evergreen tree species (Orwig and Foster 1998, Ward et al. 2004). The use of imidacloprid is critical for the preservation of this iconic foundation species.

Effects of imidacloprid treatments for HWA suppression on aquatic macroinvertebrates have been assessed by only one study (Churchel et al. 2012). Water samples and aquatic macroinvertebrate bioassessments were conducted in one control stream and four treatment streams where imidacloprid soil applications were applied in riparian areas. Imidacloprid was detected in only one water sample 720 d after imidacloprid application. Species richness and EPT (Ephemeroptera, Plecoptera, and Trichoptera) species richness did not vary between test and control streams (Churchel et al. 2012). While risks of imidacloprid use to aquatic macroinvertebrate communities were assessed, as only one water sample contained detectable levels of imidacloprid, risks to macroinvertebrate communities in the known presence of imidacloprid were not assessed.

Personnel in Great Smoky Mountains National Park (GRSM), which is located in the southern Appalachian Mountains, have implemented an extensive HWA IPM program. Eastern

hemlock ranges throughout GRSM, and occupied greater than 55,500 ha before HWA-induced mortality. Within this area more than 5,665 ha contained hemlock-dominant forests (Webster 2010). Imidacloprid soil treatments have been applied to more than 250,000 individual hemlocks, with 4,249.3 kg of imidacloprid applied to more than 4,470 ha of hemlock forests.

Since the establishment of the Park in 1934, few chemical insecticide applications have occurred. In the 1960s and 1970s DDT and Lindane were applied over 405 and 24 ha, respectively. The only recent pesticide applications in GRSM have been associated with HWA suppression. Given the limited use of insecticides in GRSM, the HWA IPM program offers a unique opportunity to assess imidacloprid effects in watersheds isolated from other chemical pollutants. Imidacloprid has been detected in streams within hemlock conservation areas where imidacloprid soil treatments were applied (Benton et al. 2016). Imidacloprid was detected in seven of ten sampled streams. The highest concentration was 0.379 µg/L, and all other detections were under 0.100 µg/L. The highest concentration detection did not exceed USEPA benchmarks. Rainfall 2 d prior to sampling eight of the streams was less than 1 cm (rainfall data were unavailable for one stream). The higher recorded rainfall amounts prior to sample collection were 2.44 and 3.35 cm (Benton et al. 2016). In an area with average precipitation of 140.30 cm/yr (US Climate Data 2015), this small amount of rainfall in 1 out of 10 streams when samples were collected indicates that most observed concentrations are representative of ambient conditions rather than high-flow storm events when higher concentrations of a pollutant would be expected.

While surface water samples provide short-term information about water quality conditions, aquatic macroinvertebrate assessments provide both short and long-term perspectives

on water quality conditions (NCDENR 2013). Presence of a taxon in a stream indicates that water quality conditions have been conducive to support the survival of that taxon. The effects of a short-term pollution event on a taxon can be observed on a long-term basis (approximately 1 yr), until the next generation of that taxon is present (NCDENR 2013).

The current study is part of a larger project to conduct a retrospective assessment of the HWA IPM program of GRSM. The purpose of this study is to assess if imidacloprid use for the suppression of HWA has negative effects on macroinvertebrate communities in streams. Imidacloprid has been detected in the majority of assessed streams in ambient conditions, so this study also provides assessments of aquatic macroinvertebrate communities in the known presence of imidacloprid in streams.

Materials and Methods

Stream Sites. Aquatic macroinvertebrate multi-habitat bioassessments were conducted at 18 sites in nine streams (one downstream site and one upstream site in each stream) that flowed through hemlock conservation areas. Imidacloprid has been detected previously in six of the nine streams sampled in the current study (Benton et al. 2016). The bioassessments were conducted to determine if imidacloprid use impacted stream water quality and biota in GRSM. Two sites were assessed in each stream: one site downstream from the imidacloprid-treated conservation area and one control site upstream of the conservation area, heretofore referred to as downstream and upstream sites, respectively (Figure 19, Table 16). Each site consisted of a 100-m stream reach. Downstream sites were selected where the stream flowed out of the

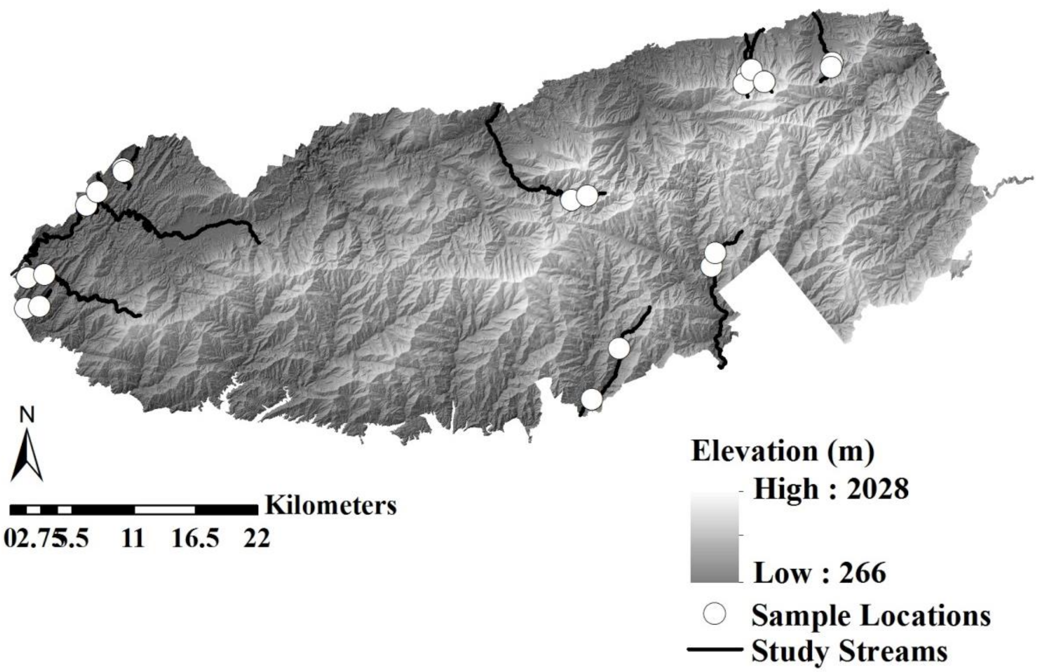


Figure 19. Sampling sites for the assessment of imidacloprid effects on aquatic macroinvertebrate communities in Great Smoky Mountains National Park.

Table 16. Macroinvertebrate bioassessment sampling dates at baseline, downstream, and upstream sites, Great Smoky Mountains National Park.

Stream	Sampling Date		
	Baseline	Downstream	Upstream
Alum Creek	19 Jul 1994	6 Aug 2012	15 Aug 2012
Camel Hump Creek	20 Aug 1996	22 Aug 2012	24 Aug 2012
Cane Creek	24 Jun 1997	19 Jun 2012	22 Jun 2012
Chasteen Creek	13 Jul 1998	12 Jul 2012	20 Jul 2012
Dunn Creek	16 Jul 1996	10 Jul 2012	17 Jul 2012
Indian Creek	20 Aug 1997	29 Aug 2012	30 Aug 2012
Kingfisher Creek	25 Jun 1997	28 Jun 2012	29 Jun 2012
Panther Creek	21 Sep 1994	21 Sep 2012	21 Sep 2012
Shop Creek	28 Sep 1994	14 Sep 2012	24 Sep 2012

conservation area, and upstream sites were a minimum of 50 m upstream from the conservation area. Water flowing through the downstream sites flowed through the entire imidacloprid-treated conservation area, thus communities located at downstream sites had the highest chance of experiencing potential impacts of imidacloprid pollution.

Hemlock conservation areas, ranging from 14.5 – 47.2 ha, contained between 100 and 1,000 hemlocks that received treatments, which were initiated 1 – 8 yr before stream sampling (see Benton et al. 2016 for additional details). Hemlocks in some conservation areas received imidacloprid treatments multiple times. All applications were made according to the product label (Bayer 2006). The limit of 0.45 kg active ingredient (AI)/ha/yr was not exceeded. Imidacloprid applications were often applied in riparian areas. However, applications were not applied within 3 m of stream banks.

Historical aquatic macroinvertebrate presence data were available from previous water quality assessments conducted between 1994 and 1997 at all downstream sites, heretofore referred to as baseline sites. These historical data serve as a baseline of species presence in streams before any environmental impacts of HWA infestations or imidacloprid use in GRSM. Downstream and upstream sampling was conducted within two weeks of the dates (day and month) that baseline samples were collected to reduce changes in macroinvertebrate communities due to seasonality (Table 16). All downstream and upstream samples were collected from June to September 2012.

Sample Collection and Identification. Rapid Bioassessment Methods (RBM) developed by the North Carolina Department of Environment and Natural Resources (NCDENR) have been used by GRSM personnel to conduct bioassessments for over 20 years (Nichols et al. 2009, NCDENR

2013). Baseline samples were conducted according to a previous, but similar, version of the current GRSM protocol (Parker and Salansky 1995). The RBM is a standardized sampling technique used by many regulatory agencies to determine water quality. Downstream and upstream samples were conducted according to current GRSM protocols (Nichols et al. 2009).

Six sampling methods (kicknet, D-net, leaf pack, rock wash, sand samples, and visual samples) were employed at each downstream and upstream site to collect from multiple stream habitats within the stream reach. Each method was repeated four times within the reach for a total of 24 individual samples collected at each site. Methods were standardized by time or area to ensure equal sampling effort between upstream and downstream sites and among the nine study streams. Kicknet (1 m^2) samples were collected by disturbing the substrate in riffles upstream from kicknet placement for 2 min. The substrate was disturbed either by kicking or moving rocks by hand, depending on the gradient of the stream. Dislodged insects flowed into the kicknet. D-net (30 cm) samples were collected in low flow areas of the stream for 1 min. Stream banks were repeatedly disturbed with the D-net, or soft substrates were disturbed by kicking. The D-net was then swept through the recently disturbed water column to collect specimens. Leaf pack samples consisted of approximately 10 cm^3 leaf packs collected from many habitats within the stream. Leaf packs were submerged in a bucket (19 L) of stream water. Leaves were agitated in the water to dislodge specimens, and then removed from the bucket. The contents of the bucket were poured through a filter to collect specimens. Ten rocks were collected evenly from areas of high and low velocity flow within the stream for rock wash samples. Rocks were rinsed in a bucket of stream water, and dislodged specimens were collected by pouring water in the bucket through a sieve. Sand samples were collected by

placing a triangle aquatic net (20 cm) downstream from an approximately 30 cm^2 area of sandy or fine gravel substrate. The substrate was disturbed by hand and specimens dislodged from the substrate were collected in the net. Contents of the net were dislodged in a bucket of stream water. The water in the bucket was poured through a filter to collect specimens. Hand collection (visual) samples were collected for 5 min. Emphasis was put on hand collecting specimens in habitats that may have been missed by other collection methods.

Samples were placed in 250 or 500 mL nalgene jars and preserved in 95% ethanol in the field. Most liquid was decanted from the samples with high organic matter content and replaced with ethanol 2 – 3 d after sample collection to ensure specimen integrity during storage. Samples were stored in the laboratory at 21°C before being processed and identified.

Samples were initially processed by removing aquatic macroinvertebrate specimens from debris in the sample and sorting to order. Due to sample volume and time constraints, three of the four repetitions of each sampling method were randomly selected for rough sorting and specimen identification. Stoneflies (Plecoptera) and caddisflies (Trichoptera), sensitive aquatic taxa, were identified to the lowest taxonomic unit given the maturity of the specimens and taxonomic key availability. The following resources were used to identify specimens: Aquatic Insects and Oligochaetes of North and South Carolina (Brigham et al. 1982), An Introduction to the Aquatic Insects of North America (Merritt et al. 2008), and a draft caddisfly key (John Morse, personal communication). Data from specimens identified to the lowest taxonomic unit for each taxon were analyzed. Specimens that were only mature enough to identify to order or family were excluded from the analyses, except in the rare case where the taxon from a family was only represented at a site by one morphospecies.

Data Analyses. All statistical tests were considered significant at $P < 0.05$. All data were stored using an Excel file (Microsoft, Redmond, WA). Sample data from the six collection methods were composited and entered into a site by species data matrix. Tolerance values, obtained from standard operating procedures from NCDNER and Tennessee Department of Environment and Conservation (TDEC), were designated for each taxon (TDEC 2011, NCDNER 2013). In addition, functional feeding groups and life habit categories were designated for each taxon. Category assignments were determined using standard operating procedures from NCDENR (NCDENR 2013) and TDEC (TDEC 2011) and Merritt et al. (2008). Functional feeding groups included collector-filterers, predators, scrapers, shredders, and generalists. Taxa with two or more functional feeding group designations were placed in the generalist category. Life habit categories were burrowers, clingers, sprawlers, and generalists. Generalists included all taxa that fit into more than one life habit category. Palaeontological Statistics Program (PAST) was used for all data analyses (Hammer 2015).

Abundance, richness, dominance, Shannon diversity, Buzas and Gibson's evenness, and mean tolerance value were used as community measures to compare all downstream and all upstream sites. Comparisons were made using t-tests ($P < 0.05$). These analyses provided overall comparisons between control sites (upstream) and downstream sites. Linear regressions were used to determine if a relationship ($P < 0.05$) between both abundance and richness and imidacloprid concentrations at each downstream site existed. Imidacloprid concentration was the predictor variable, and richness and abundance were response variables. Imidacloprid concentrations were previously determined (Benton et al. 2016).

Dominance, Shannon diversity, and evenness comparisons were made between paired downstream and upstream sites. Permutations (n = 9,999) from data from each downstream/upstream pair were generated to create a normal distribution (Hammer 2015). The distribution was based on differences in each community measure between downstream and upstream sites. Dominance, Shannon diversity, and evenness distributions were generated for each stream pair to facilitate pairwise comparisons of streams. If the observed difference of a community measure for a stream pair was below the 2.5^{th} percentile or above the 97.5^{th} percentile, then the observed difference was significantly different than what would be expected from a random comparison ($P < 0.05$). Abundance, richness, and mean tolerance value comparisons were not made, as permutations could not be generated from a single value for each site.

Correspondence analysis was used to explore relationships between all downstream and upstream sites (Quinn and Keough 2002). Correspondence analysis produces a graphical display in low-dimensional (2 axes) space of the relationships between row-column pairs, which might not be detected by pairwise analyses. Similarity of sites is indicated by close proximity of sites on the correspondence analysis graph.

Macroinvertebrate functional feeding groups and life habits in community analyses can indicate whether the trophic composition and certain life habits of communities differ between downstream and control sites (Merritt et al. 2008). Assessed categories include collector-filterers, generalists, predators, scrapers, or shredders. Collector-gatherers were not assessed as a separate group, as all collector-gatherer taxa exhibited at least two functional feeding group designations. Thus, all collector-gatherers are included within the generalist category. Life

habits assessed include burrowers, clingers, generalists, and sprawlers. All swimmer and climber taxa exhibited more than one life habit, and were included in the generalist category.

Functional feeding groups and life habits were compared between downstream and upstream sites by a t-test ($P < 0.05$). Three functional feeding group and life habit comparisons were made: abundance, richness, and proportion. Abundance was determined by the total number of individuals in each functional feeding group and each life habit category at each site. Richness was determined by the total number of taxa in each functional feeding group and each life habit category at each site. Proportion was considered as the relative percentage composition, based on abundance, of each functional feeding group and life habit category at each stream site.

Because abundance data were not available for baseline data, comparisons between baseline sites and downstream sites were limited. Community measure comparisons included mean tolerance value, richness, and richness of functional feeding groups and life habits. All comparisons between baseline and downstream sites were made by t-tests ($P < 0.05$).

Results and Discussion

Comparisons of Overall Downstream / Upstream Community Measures. During this study 10,246 stoneflies and caddisflies were collected and identified. Of the specimens collected 5,948 were mature enough to be identified to genus or species-level. Data from genus and species-level identification were used in data analyses. However, exceptions were made to include 10 specimens of one morphospecies from the caddisfly family Hydroptilidae and 26 specimens from the caddisfly family Leptoceridae in the analyses. Specimens could not be identified beyond

family. Caddisfly data included 2,123 individuals from 19 families; 50 distinct taxa were identified, 35 of which were species-level identifications. Stonefly data included 3,825 individuals; 16 distinct stonefly taxa from seven families were identified. However, due to immaturity of specimens or lack of species level keys, only three stonefly taxa were identified to species level.

Abundance ranged from 57 – 662 individuals collected from each site (Table 17). Abundance of stoneflies and caddisflies at each downstream site was not related to imidacloprid concentrations detected at downstream sites (P = 0.683, R^2 = 0.03) (Benton et al. 2016) (Figure 20). Streams where higher concentrations of imidacloprid were detected in a one-time sampling event did not have lower abundance. Mean abundance for all downstream and all upstream sites was 360.780 and 300.110, respectively. Abundance was not significantly different between downstream and upstream sites (P = 0.496) (Table 18).

Taxa richness at stream sites ranged from 11 – 26 (Table 17). Richness at downstream sites also was not related to observed imidacloprid concentrations in the sampled streams (P = 0.205, R^2 = 0.22) (Figure 21). Detected concentrations of imidacloprid, which were below USEPA thresholds, did not result in low species richness where higher concentrations were present. In fact, Dunn Creek, the site with the highest recorded imidacloprid concentration (Benton et al. 2016), also had the highest taxa richness. Mean downstream and upstream richness was 18.330 and 16.333, respectively. Richness did not significantly differ between downstream and upstream sites (P = 0.363).

Table 17. Stonefly and caddisfly richness and abundance in streams in Great Smoky Mountains National Park, 2012.

Stream	Abundance[1]		Richness[2]		
	Downstream[3]	Upstream[4]	Baseline[5]	Downstream	Upstream
Alum Creek	397	321	13	16	19
Camel Hump Creek	529	344	26	18	15
Cane Creek	394	348	21	24	19
Chasteen Creek	396	398	18	22	20
Dunn Creek	488	147	21	26	15
Indian Creek	647	662	25	21	19
Kingfisher Creek	309	270	18	13	17
Panther Creek	71	154	21	14	20
Shop Creek	76	57	16	11	13

[1]Abundance is the total number of individuals collected at each site.
[2]Richness is the total number of taxa collected at each site.
[3]Sites downstream from imidacloprid-treated conservation areas.
[4]Sites upstream from conservation areas.
[5]Baseline data collected from each downstream location between 1994 – 1997.

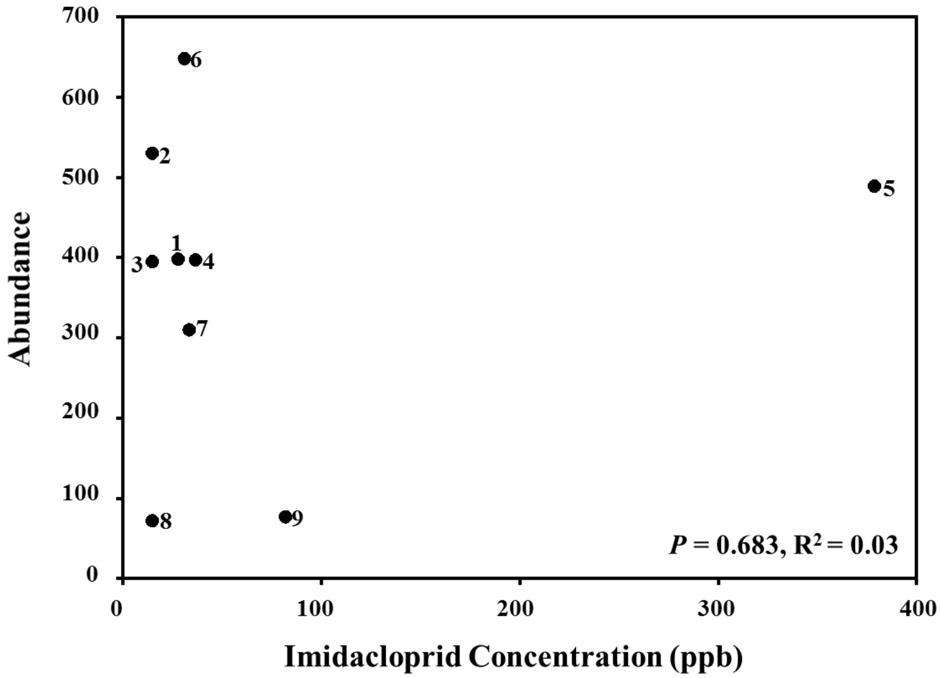


Figure 20. Regression analysis of imidacloprid concentrations at downstream sites and stonefly and caddisfly abundance.

Number labels represent streams names: Alum Creek (1), Camel Hump Creek (2), Cane Creek (3), Chasteen Creek (4), Dunn Creek (5), Indian Creek (6), Kingfisher Creek (7), Panther Creek (8), and Shop Creek (9).

Table 18. Comparisons of stonefly and caddisfly community measures between downstream and upstream sampling sites, Great Smoky Mountains National Park, 2012.

Community Measure	Downstream		Upstream		P Value[1]
	Mean[2]	95% Confidence Interval	Mean[3]	95% Confidence Interval	
Abundance	360.780[4]	212.606 - 508.900	300.110	164.230 - 435.990	0.496
Richness	18.330	14.350 - 22.350	16.333	13.483 - 19.185	0.363
Dominance	0.240	0.154 - 0.327	0.269	0.118 - 0.421	0.707
Evenness	0.409	0.287 - 0.532	0.447	0.283 - 0.611	0.678
Shannon Diversity	1.923	1.658 - 2.188	1.829	1.418 - 2.239	0.662
Tolerance Value	1.746	1.501 - 1.990	1.598	1.392 - 1.804	0.302

[1] $P < 0.05$, t-test

[2] Mean of each community measure from all downstream sites.

[3] Mean of each community measure from all upstream sites.

[4] There were no significant differences in community measures of downstream and upstream sites.

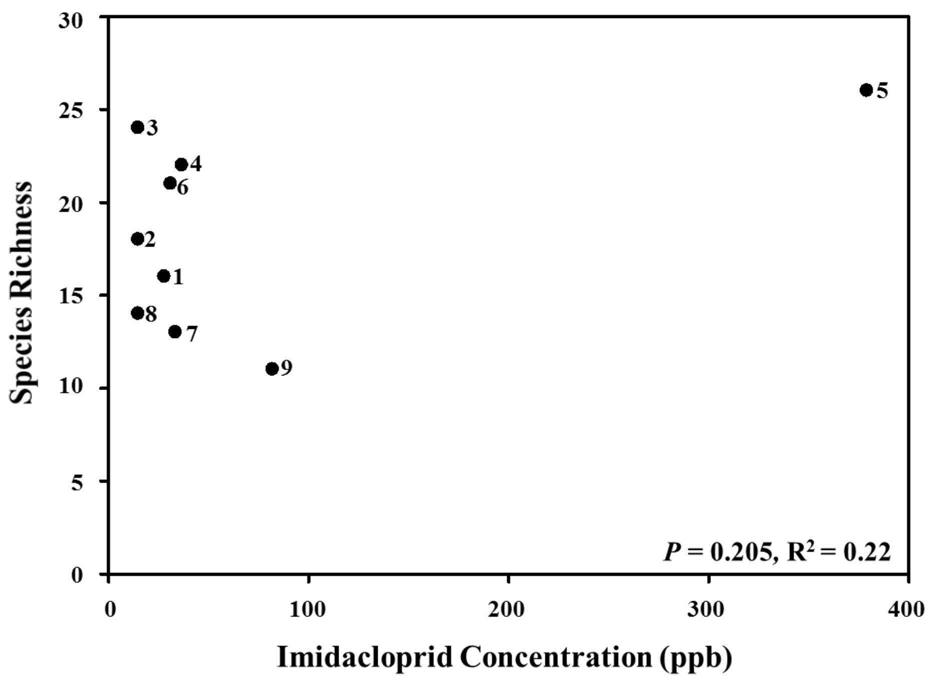


Figure 21. Regression analysis of imidacloprid concentrations at downstream sites and stonefly and caddisfly species richness.

Number labels represent streams names: Alum Creek (1), Camel Hump Creek (2), Cane Creek (3), Chasteen Creek (4), Dunn Creek (5), Indian Creek (6), Kingfisher Creek (7), Panther Creek (8), and Shop Creek (9).

Higher dominance values indicate a community that is dominated by a single taxon, whereas higher evenness values indicate a community with more even abundance distribution among numerous taxa. High Shannon diversity is characteristic of a more diverse community (Magurran 2004). Thus, a more diverse community would have lower dominance, higher evenness, and a higher Shannon diversity index.

Dominance, evenness, and Shannon diversity community measures indicated similar communities at downstream compared to upstream sites. Dominance was 0.240 and 0.269 for downstream and upstream sites, respectively (Table 18), indicating that communities were not dominated by a single taxon. Evenness, 0.409 and 0.447 for downstream and upstream sites, respectively (Table 18), indicated that these communities have moderately even abundance distributions among taxa. Downstream and upstream sites did not differ in dominance (P = 0.707) or evenness (P = 0.678) (Table 18). Shannon diversity, which was 1.923 and 1.829 at downstream and upstream sites, respectively, was not significantly different between downstream and upstream sites (P = 0.662).

Tolerance values, which are scaled from 0 – 10, indicate the ability of a taxon to survive in stressful water quality conditions. Lower values indicate an intolerant taxon that requires pristine water quality for survival, and higher values indicate a tolerant taxon that can survive in poor water quality.

Low mean tolerance values at downstream (1.746) and upstream (1.598) sites indicate that the stonefly and caddisfly communities are comprised of taxa that are intolerant to poor water quality conditions (Table 18). Mean tolerance values did not differ between downstream and upstream sites (P = 0.302). Presence of pesticide concentrations harmful to aquatic

communities would prohibit the survival of intolerant indicator taxa. Only two taxa had tolerance values above 5.0. Hydroptilidae (Trichoptera: Hydroptilidae) (n = 10) has a tolerance value of 6.5 and was only collected from one upstream and one downstream site. As hydroptilid specimens were only identified to family, the highest tolerance value assigned to a genus in the family was used in analyses as a conservative approach. *Cheumatopsyche* spp. (Trichoptera: Hydropsychidae) (n = 143) has a tolerance value of 6.6 and was only collected at three downstream and two upstream sites. However, *Tallaperla* spp. (Plecoptera: Peltoperlidae), the most abundant taxon (n = 2,171), has a tolerance value of 1.3 and was collected at every site. If taxa at downstream sites were affected by poor water quality, it would be expected that mean tolerance values of taxa at downstream sites would be higher than those at upstream control sites. Taxa with higher tolerance values at downstream sites have not been observed. Predominance of tolerant taxa is a sign of compromised water quality (CCME 2007). However, few tolerant taxa were collected, and the most abundant taxon has a low tolerance value.

Comparisons of Pairwise Downstream / Upstream Community Measures. Pairwise comparisons of community measures were made between dominance, evenness, and Shannon diversity at downstream and upstream sites of each stream to assess potential imidacloprid impacts in individual streams. Camel Hump Creek, Chasteen Creek, and Panther Creek had similar dominance, evenness, and Shannon diversity between downstream and upstream sites ($P > 0.05$) (Table 19). Indian Creek had mixed results of more diverse community measures at downstream and upstream sites. Lower dominance and higher Shannon diversity was detected at the downstream site ($P < 0.001$), indicating a more diverse downstream community. However,

Table 19. Pairwise comparisons of stonefly and caddisfly community measures between downstream and upstream sampling sites, Great Smoky Mountains National Park, 2012.

Stream and Community Measure	Downstream Value[1]	Downstream 95% Confidence Interval[2]	Upstream Value	Upstream 95% Confidence Interval	*P* Value[3]
Alum Creek					
Dominance	0.237a[4]	0.213 - 0.258	0.259a	0.237 -0.286	0.224
Evenness	0.361a	0.329 - 0.408	0.529b	0.481 - 0.576	< 0.001
Shannon Diversity	1.753a	1.661 - 1.875	1.560a	1.465 - 1.645	0.040
Camel Hump Creek					
Dominance	0.199a	0.181 - 0.218	0.182a	0.162 - 0.204	0.249
Evenness	0.386a	0.358 - 0.422	0.500a	0.452 - 0.550	0.055
Shannon Diversity	1.938a	1.863 - 2.028	2.014a	1.915 - 2.111	0.337
Cane Creek					
Dominance	0.200a	0.172 – 0.221	0.273b	0.231 – 0.310	< 0.001
Evenness	0.326a	0.299 – 0.375	0.311a	0.285 – 0.363	0.746
Shannon Diversity	2.064a	1.97 – 2.20	1.790b	1.690 – 1.932	0.004
Chasteen Creek					
Dominance	0.317a	0.265 - 0.358	0.256a	0.217 - 0.286	0.050
Evenness	0.278a	0.247 - 0.325	0.316a	0.291 - 0.363	0.249
Shannon Diversity	1.810a	1.692 - 1.967	1.842a	1.762 - 1.983	0.767
Dunn Creek					
Dominance	0.151a	0.128 -0.171	0.206b	0.162 - 0.245	0.023
Evenness	0.423a	0.388 - 0.473	0.473a	0.422 - 0.567	0.922
Shannon Diversity	2.397a	2.311 - 2.509	1.958b	1.845 - 2.140	0.001
Indian Creek					
Dominance	0.209a	0.190 - 0.224	0.767b	0.713 - 0.798	< 0.001
Evenness	0.326a	0.307 - 0.361	0.104b	0.097 - 0.120	< 0.001
Shannon Diversity	1.924a	1.862 - 2.024	0.685b	0.611 - 0.823	< 0.001
Kingfisher Creek					
Dominance	0.501a	0.432 – 0.564	0.27b	0.233 - 0.294	< 0.001
Evenness	0.275a	0.236 - 0.321	0.316a	0.291 - 0.372	0.279
Shannon Diversity	1.273a	1.119 - 1.431	1.681b	1.599 - 1.844	< 0.001

Table 19. Continued.

Stream and Community Measure	Downstream		Upstream		P Value[3]
	Value[1]	95% Confidence Interval[2]	Value	95% Confidence Interval	
Panther Creek					
Dominance	0.115a[4]	0.095 - 0.163	0.105a	0.088 - 0.131	0.544
Evenness	0.780a	0.633 - 0.851	0.642a	0.558 - 0.717	0.112
Shannon Diversity	2.390a	2.181 - 2.477	2.552a	2.412 - 2.663	0.290
Shop Creek					
Dominance	0.237a	0.182 - 0.288	0.108b	0.095 - 0.154	< 0.001
Evenness	0.531a	0.452 - 0.654	0.831b	0.676 - 0.888	< 0.001
Shannon Diversity	1.765a	1.603 - 1.973	2.379b	2.172 - 2.446	0.002

[1]Value of each community measure for each stream site.
[2]95% confidence interval of each community measure generated by a permutation process (n = 9,999).
[3]$P < 0.05$, based on difference between downstream and upstream community measures compared to random distribution of differences generated by a permutation process.
[4]Means within a row followed by the same letters are not significantly different ($P < 0.05$, t-test).

evenness was significantly lower at the upstream site ($P < 0.001$), which is indicative of a less diverse community.

Alum Creek, Cane Creek, and Dunn Creek community measure analyses show higher diversity communities at the downstream sites. Evenness was lower and Shannon diversity was higher at Alum Creek ($P < 0.001$ and $P = 0.040$, respectively) (Table 19). Dominance was lower and Shannon diversity was higher at the downstream site of Cane Creek ($P < 0.001$ and $P = 0.004$, respectively). Dunn Creek, the site with the highest recorded imidacloprid concentration (Benton et al. 2016), had lower dominance and higher Shannon diversity at the downstream site ($P = 0.023$ and $P < 0.001$, respectively).

Kingfisher Creek and Shop Creek community measures indicate higher diversity communities at upstream sites. Dominance was lower and Shannon diversity was higher at the upstream site of Kingfisher Creek ($P < 0.001$ and $P < 0.001$, respectively) (Table 19). Shop Creek has a more diverse community at the upstream site. Dominance and evenness were lower ($P < 0.001$ and $P < 0.001$, respectively). Shannon diversity was higher at the upstream site ($P = 0.002$).

Differences in community measures between downstream and upstream sites are not completely unexpected. Downstream and upstream pairs were separated by approximately 350 – 8,700 m of stream length flowing through conservation areas. Streams flow through the diverse forests of GRSM, with a variety of inputs between downstream and upstream sites. While some differences in paired sites of individual streams are expected, due to stochastic effects in the environment, overall trends of lower community diversity in downstream sites would be concerning. However, the results of pairwise community analyses did not show this effect.

Pairwise comparisons showed a mix of both upstream and downstream sites with higher diversity community measures, as well as three streams with no difference between upstream and downstream pairs. Detrimental effects in downstream sites that could be attributed to imidacloprid contamination were not observed.

Downstream / Upstream Correspondence Analysis. Correspondence analysis based on abundance data in a site by species matrix shows similarity between most downstream and upstream site pairs (Figure 22). Based upon community similarity, the communities of Panther Creek and Shop Creek are distinctive from the other communities. The presence of distinctive communities at Panther Creek and Shop Creek is not surprising, given that the streams are located in lower gradient areas of GRSM and contain greater amounts of sandy substrate compared to other streams. Downstream communities at both Alum Creek and Dunn Creek are distinctive when compared to their corresponding upstream communities. While community measures in pairwise comparisons of downstream and upstream sites at Alum and Dunn Creek differ, both of these streams have higher diversity downstream communities. Distinctiveness of these downstream and upstream communities is likely not related to imidacloprid impacts, but to a myriad of other environmental factors in the diverse landscape through which the streams flow.

Downstream / Upstream Functional Feeding Groups. Similar abundance of collector-filterers, generalists, predators, scrapers, and shredders was observed between upstream and downstream sites ($P = 0.291$, $P = 0.837$, $P = 0.677$, $P = 0.136$, and $P = 0.649$, respectively) (Table 20). Communities in both downstream and upstream sites had high generalist abundance and low predator abundance. Collector-filterers, scrapers, and shredders were moderately abundant in comparison. Functional feeding group taxa richness ranged from 2.111 – 4.778.

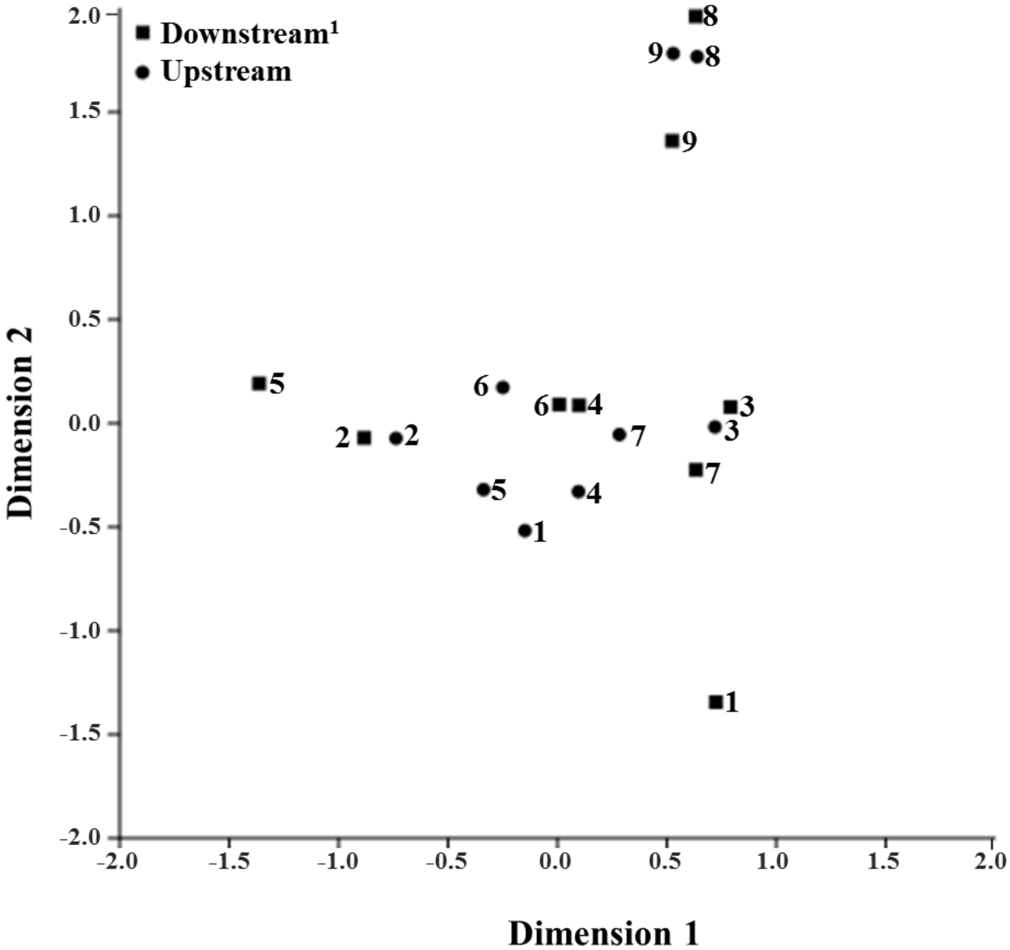


Figure 22. Graphical representation of community similarity by correspondence analysis of combined stonefly and caddisfly species richness and abundance from streams in Great Smoky Mountains National Park.

(Sites located more closely together on the figure have more similar communities)
[1]Alum Creek (1), Camel Hump Creek (2), Cane Creek (3), Chasteen Creek (4), Dunn Creek (5), Indian Creek (6), Kingfisher Creek (7), Panther Creek (8), and Shop Creek (9).

Table 20. Comparisons of stonefly and caddisfly functional feeding groups between downstream and upstream sampling sites, Great Smoky Mountains National Park, 2012.

Community Measure	Downstream		Upstream		*P* Value[3]
	Mean[1]	95% Confidence Interval	Mean[2]	95% Confidence Interval	
Abundance					
Collector-filterers	49.333[4]	26.996 - 71.671	33.889	10.137 - 57.641	0.291
Generalists	137.560	46.54 - 228.57	152.560	14.218 - 290.89	0.837
Predators	11.667	5.043 - 18.290	9.889	2.850 - 16.928	0.677
Scrapers	46.222	12.204 - 80.241	21.778	10.333 - 33.223	0.136
Shredders	75.667	15.425 - 135.910	61.000	20.08 - 101.920	0.649
Richness					
Collector-filterers	4.222	3.298 - 5.146	3.333	2.118 - 4.549	0.198
Generalists	4.778	3.300 - 6.255	4.778	3.576 - 5.980	0.999
Predators	2.889	1.647 - 4.131	2.667	1.580 - 3.754	0.760
Scrapers	3.556	2.878 - 4.233	3.444	2.107 - 4.782	0.866
Shredders	2.778	1.639 - 3.916	2.111	1.301 - 2.921	0.288

Table 20. Continued.

Community Measure	Downstream		Upstream		P Value[3]
	Mean[1]	95% Confidence Interval	Mean[2]	95% Confidence Interval	
Proportion					
Collector-filterers	0.203[4]	0.091 - 0.315	0.165	0.053 - 0.277	0.588
Generalists	0.383	0.211 - 0.555	0.457	0.267 - 0.648	0.512
Predators	0.050	0.012 - 0.088	0.044	0.017 - 0.071	0.773
Scrapers	0.141	0.077 - 0.205	0.107	0.048 - 0.166	0.381
Shredders	0.219	0.041 - 0.397	0.227	0.112 - 0.342	0.928

[1]Mean of each community measure from all downstream sites.
[2]Mean of each community measure from all upstream sites.
[3]$P < 0.05$, t-test
[4]There was no significant difference in community measures between downstream and upstream sites.

Taxa richness of collector-filterers, generalists, predators, scrapers, and shredders did not vary between downstream and upstream sites (*P* = 0.198, *P* = 0.999, *P* = 0.760, *P* = 0.866, and *P* = 0.288, respectively). In addition, downstream and upstream sites had similar proportions of collector-filterers, generalists, predators, scrapers, and shredders (*P* = 0.588, *P* = 0.512, *P* = 0.773, *P* = 0.381, and *P* = 0.928, respectively). Given that abundance, richness, and proportion of functional feeding groups do not differ between downstream and upstream sites, imidacloprid use in GRSM is not affecting trophic composition of stonefly and caddisfly communities.

Downstream / Upstream Life Habits. Clinger abundance was 125.330 and 72.222 at downstream and upstream sites, respectively. Generalist abundance was 183.670 and 204.670 at downstream and upstream sites, respectively. Burrower and sprawler abundance was much lower. No differences in the abundance of burrowers, clingers, generalists, and sprawlers between downstream and upstream sites were detected (*P* = 0.536, *P* = 0.120, *P* = 0.758, and *P* = 0.304, respectively) (Table 21). Burrower, clinger, and generalist richness did not vary between downstream and upstream sites (*P* = 0.999, *P* = 0.539, and *P* = 0.914, respectively). Sprawler richness was significantly higher at downstream sites compared to upstream sites (*P* = 0.035). Clingers and generalists made up the highest proportions of downstream and upstream communities. Sprawlers only comprised 3.8% of the taxa at downstream sites. The proportion of burrowers, clingers, generalists, and sprawlers did not differ between downstream and upstream sites (*P* = 0.352, *P* = 0.335, *P* = 0.270, and *P* = 0.486, respectively). Overall, abundance and richness of taxa with different life habits from downstream and upstream communities were

Table 21. Comparisons of stonefly and caddisfly life habits between downstream and upstream samping sites, Great Smoky Mountains National Park, 2012.

Community Measure	Downstream		Upstream		*P* Value[3]
	Mean[1]	95% Confidence Interval	Mean[2]	95% Confidence Interval	
Abundance					
Burrowers	0.111a[4]	-0.145 - 0.367	0.333a	-0.435 - 1.102	0.536
Clingers	125.330a	59.931 - 190.74	72.222a	36.253 - 108.190	0.120
Generalists	183.670a	107.56 - 259.780	204.670a	70.184 - 339.15	0.758
Sprawlers	11.111a	8.794 - 31.016	1.889a	-0.197 - 3.974	0.304
Richness					
Burrowers	0.111a	-0.145 - 0.367	0.111a	-0.145 - 0.367	0.999
Clingers	10.444a	8.167 - 12.722	9.556a	7.214 - 11.897	0.539
Generalists	6.222a	4.197 - 8.248	6.111a	4.930 - 7.292	0.914
Sprawlers	1.333a	0.789 - 1.877	0.556b	-0.003 - 1.114	0.035
Proportion					
Burrowers	0.000a		0.006a	-0.008 - 0.019	0.352
Clingers	0.429a	0.268 - 0.591	0.329a	0.162 - 0.496	0.335
Generalists	0.533a	0.390 - 0.675	0.648a	0.464 - 0.831	0.270
Sprawlers	0.038a	-0.021 - 0.097	0.017a	-0.013 - 0.048	0.486

[1]Mean of each community measure from all downstream sites.
[2]Mean of each community measure from all upstream sites.
[3]$P < 0.05$, t-test.
[4]Means within a row followed by the same letters are not significantly different.

similar, with the exception of sprawler richness. Imidacloprid use did not negatively affect the abundance, richness, or proportion of taxa with different life habits at downstream sites.

Downstream / Baseline Comparison. Since baseline data were limited to presence/absence data, analyses were limited to mean tolerance value and richness comparisons. Mean downstream and baseline richness, 18.333 and 19.667, respectively, were not significantly different (P = 0.558) (Table 22). Baseline mean tolerance value was low at 1.636, indicating that baseline stonefly and caddisfly communities were comprised of taxa that were intolerant to poor water quality. Mean tolerance value did not significantly vary between downstream and baseline sites (P = 0.472).

Functional feeding group richness in baseline sites ranged from 2.444 – 4.778. Collector-filterer, generalist, and shredder richness values were not significantly different between downstream and baseline stream sites (P = 0.365, P = 0.999, and P = 0.540, respectively) (Table 22). Baseline predator richness (5.000) was significantly higher (P = 0.023) than downstream predator richness (2.889). Scraper richness, 3.556 and 2.444 at downstream and baseline sites, respectively, was significantly higher at downstream sites (P = 0.005). Functional feeding group richness did differ between downstream and baseline sites. Since higher predator and scraper functional feeding group richness was observed at both downstream and baseline sites, respectively, richness differences do not clearly indicate impaired communities at downstream sites.

Life habits were similar between downstream and upstream communities. Burrower richness at all baseline sites was 0 (i.e., no burrowers were collected), so burrower richness could not be analyzed. Downstream burrower richness was only 0.111. Clinger, generalist, and

Table 22. Comparisons of stonefly and caddisfly community measures between downstream and baseline sampling sites, Great Smoky Mountains National Park, 1994 – 1997 and 2012.

Community Measure	Downstream		Baseline		*P* Value[3]
	Mean[1]	95% Confidence Interval	Mean[2]	95% Confidence Interval	
Mean Tolerance Value	1.746[4]	1.501 - 1.990	1.636	1.392 - 1.879	0.472
Richness	18.333	14.321 - 22.346	19.667	16.451 - 22.882	0.558
Richness					
Collector-filterers	4.222	3.298 - 5.146	3.667	2.650 - 4.684	0.365
Generalists	4.778	3.330 - 6.255	4.778	4.031 - 5.525	0.999
Predators	2.889	1.647 - 4.131	5.000	3.512 - 6.489	0.023
Scrapers	3.556	2.787 - 4.233	2.444	2.040 - 2.850	0.005
Shredders	2.778	1.639 - 3.916	3.111	2.649 - 3.573	0.540
Richness					
Burrowers	0.111	-0.145 - 0.367	0		N/A[5]
Clingers	10.444	8.167 - 12.722	11.000	8.662 - 13.338	0.700
Generalists	6.222	4.197 - 8.248	7.111	6.301 - 7.921	0.361
Sprawlers	1.333	0.790 - 1.877	1.111	0.398 - 1.724	0.576

[1]Mean of each community measure from all downstream sites.
[2]Mean of each community measure from all baseline sites.
[3]$P < 0.05$, t-test.
[4] There were no significant differences in community measures of downstream and baseline sites.
[5]Since burrower proportion at downstream sites was 0, there was no variation in the data, and a t-test could not be performed.

sprawler richness values were similar at downstream and baseline stream sites ($P = 0.700$, $P = 0.361$, and $P = 0.576$, respectively) (Table 22). The similarity in tolerance values, functional feeding groups, and life habits of taxa at downstream and baseline sites indicates that water quality conditions did not vary dramatically in the time between baseline sample collection in the mid-1990s and downstream collections in 2012 after the implementation of the HWA IPM program.

Conclusions

Given global concerns about non-target impacts of neonicotinoids, it is important that pest management programs do not cause undue risks to ecosystems. Concern for reduction of non-target impacts is especially relevant for management programs, such as the HWA IPM program at GRSM, operating with the objective of ecosystem preservation. Imidacloprid is used to preserve systems such as these, where often pest suppression can be maintained for multiple years using one treatment (Coots 2012, Coots et al. 2013, Eisenback et al. 2014, Benton et al. 2015). It is important to recognize that imidacloprid use in these systems is inherently different than use in agricultural systems where imidacloprid and numerous other pesticides are used on an annual basis.

This research is the first study to assess potential effects of imidacloprid on aquatic macroinvertebrate communities in natural settings, in the known presence of imidacloprid, where other pollutants were not confounding factors. The assessment of the GRSM HWA IPM program demonstrates that stonefly and caddisfly communities downstream from hemlock conservation areas are similar to baseline conditions and upstream controls. Imidacloprid

concentrations detected in surface waters in GRSM did not exceed USEPA environmental standards, however detected concentrations did exceed guidelines set by the Netherlands and Canada. Sites downstream from conservation areas are typified by rich and diverse stonefly and caddisfly communities, with taxa that are intolerant to poor water quality conditions. The use of imidacloprid for HWA suppression in forest settings has not had a negative effect on stonefly and caddisfly communities. Results of this study and Benton et al. (2016) demonstrate that imidacloprid, when used within the limits of USEPA federal regulations, has not had detrimental impacts to aquatic macroinvertebrates.

CHAPTER VII. CONCLUSIONS

The loss of eastern hemlocks in eastern forests is causing cascading environmental effects. Many government agencies and private landowners work to minimize the damage of HWA in forest settings. Resource managers must balance hemlock conservation with budgetary restrictions, while minimizing nontarget risks. The reduction of nontarget risks is especially important in light of public concern over the use of neonicotinoid pesticides and pending shifts in federal policy. Resource managers need guidance to optimize their programs for maximum hemlock conservation while minimizing environmental risks. The outcome of this project is guidance on the longevity of imidacloprid treatments, a new option for treating different size hemlocks, and information on nontarget risks in aquatic systems.

Imidacloprid treatments have greater longevity and efficacy in hemlocks than originally thought due to the action of the insecticidal olefin metabolite. Both imidacloprid and olefin are present in hemlock foliage 4 – 7 yr after a single imidacloprid treatment. Imidacloprid concentrations were below the lethal concentration (LC_{50}) for HWA 4 - 7 yr post-treatment. Olefin concentrations were only above the LC_{50} 4 yr post-treatment. However, peak effects of imidacloprid suppression of HWA were observed 5 yr post-treatment, when imidacloprid and olefin concentrations were below the LC_{50} for HWA. Several scenarios may be responsible for the observed efficacy of imidacloprid treatments in the absence of seemingly sufficient concentrations of insecticidal compounds. Possible scenarios include lower LC_{50} for HWA in field conditions, additive effects of residual imidacloprid and olefin concentrations, and cumulative effects of reduced HWA populations over numerous years.

Hemlock canopy health was not affected by variations in the levels of HWA populations, meaning that HWA populations were sufficiently suppressed to maintain hemlock health for up

to 7 yr post-treatment. Instead of routine treatments every 5 yr, assessing treated hemlocks for infestation levels at 5 yr, with the option of delaying retreatments up to 7 yr, can offer financial benefits and reduced environmental risks, through less frequent pesticide applications, to hemlock management programs.

Imidacloprid and olefin concentrations varied among different DBH size class hemlocks. Larger DBH hemlocks, which were given higher doses of imidacloprid, exhibited higher concentrations of imidacloprid and olefin in most canopy strata compared to 45 cm hemlocks. Concentrations of olefin in larger hemlocks were still above LC_{50} for HWA, while smaller trees had concentrations below the LC_{50}. The current treatment recommendation of increasing the dose of imidacloprid at 63.5 cm DBH resulted in smaller hemlocks needing reapplication of imidacloprid before larger hemlocks. A model for the optimization of imidacloprid treatments based on hemlock DBH was developed to increase efficiency in imidacloprid treatments, with the goal of all DBH size classes being ready for reapplication at the same time. The model formula would result in hemlocks < 30 cm and > 63 cm DBH receiving lower dosage treatments than current recommendations. The expected persistence of olefin in foliage from this treatment plan would result in > 80% HWA population reduction in each generation for 4 – 6 yr post-treatment. The proposed management plan results in both an increase in the efficacy duration and the reduction of insecticide application amounts, conveying financial and environmental benefits to resource managers.

The use of imidacloprid for HWA suppression in the pristine watersheds of GRSM offered a rare opportunity to assess imidacloprid risks to water quality and aquatic macroinvertebrates in isolation from other known pollutants. Imidacloprid is present in surface

waters of GRSM as a result of imidacloprid treatments. Detections of imidacloprid in seven of ten sampled streams were below USEPA Aquatic Life Benchmarks for acute and chronic toxicity for aquatic macroinvertebrates. The amount of imidacloprid applied to a watershed was positively related to the amount of imidacloprid present in streams.

Bioassessments of stonefly and caddisfly communities showed no detectable negative impacts to these sensitive taxa as a result of imidacloprid use. Communities upstream and downstream from conservation areas were similar in diversity and function. Aquatic macroinvertebrate communities downstream from conservation areas were similar to communities before imidacloprid use in the watersheds. Imidacloprid use for HWA suppression in GRSM has resulted in no detectable impairments to sensitive aquatic macroinvertebrate communities and concentrations of imidacloprid in surface waters that are within USEPA guidelines. These results exhibit the safety of the use of imidacloprid in forest settings when applications are made within the limits set by the product label.

Knowledge of the persistence, efficacy, and nontarget risks of imidacloprid use in mixed DBH size hemlocks for up to 7 yr post-treatment can assist resource managers in their conservation efforts. Imidacloprid treatments can be extended for up to 7 yr between treatments, and dosages can be optimized based on the diameter of the hemlock, resulting in more efficient use of insecticides. In addition, the current use of imidacloprid in forest settings has resulted in no observable detrimental effects to sensitive aquatic taxa. Treatment recommendations and increased knowledge of nontarget risks will result in the reduction of imidacloprid use, continued quality of HWA suppression, and confidence that risks to aquatic communities are minimal.

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