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Environmental safety to decomposer invertebrates of azadirachtin (neem) as a systemic insecticide in trees to control emerald ash borer

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ABSTRACT

The non-target effects of an azadirachtin-based systemic insecticide used for control of wood-boring insect pests in trees were assessed on litter-dwelling earthworms, leaf-shredding aquatic insects, and microbial communities in terrestrial and aquatic microcosms. The insecticide was injected into the trunks of ash trees at a rate of 0.2 g azadirachtin cm^{-1} tree diameter in early summer. At the time of senescence, foliar concentrations in most (65%) leaves where at or below detection (<0.01 mg kg^{-1} total azadirachtin) and the average concentration among leaves overall at senescence was 0.19 mg kg^{-1}. Leaves from the azadirachtin-treated trees at senescence were added to microcosms and responses by test organisms were compared to those in microcosms containing leaves from non-treated ash trees (controls). No significant reductions were detected among earthworm survival, leaf consumption rates, growth rates, or cocoon production, aquatic insect survival and leaf consumption rates, and among terrestrial and aquatic microbial decomposition of leaf material in comparison to controls. In a further set of microcosm tests containing leaves from intentional high-dose trees, the only significant, adverse effect detected was a reduction in microbial decomposition of leaf material, and only at the highest test concentration ($\sim$6 mg kg^{-1}). Results indicated no significant adverse effects on litter-dwelling earthworms or leaf-shredding aquatic insects at concentrations up to at least 30 $\times$ the expected field concentrations at operational rates, and at 6 $\times$ expected field concentrations for adverse effects on microbial decomposition. We conclude that when azadirachtin is used as a systemic insecticide in trees for control of insect pests such as the invasive wood-boring beetle, emerald ash borer, resultant foliar concentrations in senescent leaf material are likely to pose little risk of harm to decomposer invertebrates.

1. Introduction

The emerald ash borer (*Agrilus planipennis*) is an exotic, invasive wood-boring insect pest in North America that is causing extensive ash tree (*Fraxinus* spp.) mortality across an area extending from southwestern Ohio, USA to southwestern Quebec, Canada, and is spreading rapidly (Cappaert et al., 2005). Owing to its rapid spread, the difficulties in early detection, and the scarcity of natural pathogens, predators and parasites, this invasive beetle poses a threat of nearly complete loss of ash trees from urban and rural landscapes with consequential ecological and economic impacts (Poland and McCullough 2006; Gandhi and Herms, 2010; Kovacs et al., 2010). For example, Kovacs et al. (2010) estimate the cost of dealing with dead ash trees over the next decade in USA urban settings alone will exceed 10 billion dollars, while the ecological costs could include the long-term disruption and cascade of changes in forest ecosystem structure and the loss or reduction in some fundamental ecosystem processes (Ellison et al., 2005).

Among the options being explored to slow the spread of the emerald ash borer and other wood-boring insect pests is the use of systemic insecticides to protect high-value trees and stands, and to reduce the source of adult (mobile) beetles from infested trees (Poland et al., 2006; McKenzie et al., 2010; Mercader et al., 2011). Systemic trunk injections of insecticides offer the environmental benefit of specific, targeted applications to reduce overall environmental exposure, and may be particularly well suited for environmentally sensitive areas such as riparian (shoreline) forests, wooded wetlands, urban parks and conservation areas. However, systemic injections in trees will result in foliar insecticide concentrations that could pose a risk of harm to non-target decomposer organisms when autumn-shed leaves fall to forest floors or adjacent water bodies. Given that invertebrate-mediated decomposition of leaf litter is a critical forest and aquatic ecosystem

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process (Graça, 2001; Hattenschwiler et al., 2005), adverse insecticide impacts on decomposer invertebrates could have ecologically significant implications for leaf litter breakdown and nutrient cycling in detritus-based food webs of forest floors and nearby aquatic ecosystems. The risk of harmful insecticide effects on decomposer organisms and processes will depend on the level of exposure (the insecticide concentrations in leaves at senescence and number of trees treated in a given area) and on the toxicity of the insecticide at those concentrations.

One of the systemic insecticides that is registered and recommended for emerald ash borer in the USA is the chloro-neonicotinyl insecticide, imidacloprid (Poland et al., 2006; Smitley et al., 2010). We previously showed that when imidacloprid was applied to trees for wood-borer control, it resulted in foliar concentrations at senescence that significantly reduced the decomposition of leaf litter in terrestrial and aquatic microcosms (Kreutzweiser et al., 2007, 2008). We further determined that the reduced litter decomposition resulted from sub-lethal, antifeedent effects on litter-dwelling earthworms and leaf-shredding aquatic insects, and from significant inhibition of terrestrial and aquatic microbial decomposition activity (Kreutzweiser et al., 2009). Those studies suggested that imidacloprid may not be the best option for a systemic insecticide in environmentally sensitive areas because of its potential adverse effects on decomposition processes.

As an alternative to imidacloprid, an azadirachtin-based systemic insecticide is being evaluated in Canada for control of emerald ash borer and other insects (Helson et al., 2001; McKenzie et al., 2010). Azadirachtin is a natural tetranortriterpenoid compound derived from seed kernels of the neem tree (*Azadirachta indica*) that has been shown to express insecticidal activity and be highly effective against a number of insect pests (Schmutterer, 1990; Ascher, 1993). Although the toxicological profile of azadirachtin is generally favorable (Boeke et al., 2004; Stark, 2007; Thompson and Kreutzweiser, 2007), its potential for non-target effects has not been assessed in the context of a systemic insecticide for forest insect pests. Given the rapid uptake and translocation of azadirachtin in trunk-injected ash trees and the presence of foliar concentrations at biologically active concentrations (McKenzie et al., 2010), we hypothesized that azadirachtin as a systemic insecticide could pose measurable risk of harm to non-target decomposer invertebrates at realistic foliar concentrations, similar to our findings for the imidacloprid assessment. Therefore, following similar field applications and microcosm experiments to those used previously for our assessment of imidacloprid, we determined the effects of leaves collected from azadirachtin-treated trees at senescence on litter-dwelling earthworms, leaf-shredding aquatic insects, and microbial community decomposition activity in terrestrial and aquatic microcosms.

2. Materials and Methods

2.1. Experimental treatments

Two separate microcosm experiments were conducted following the same protocols but using leaves from separate tree injections. The first experiment (Experiment 1) was conducted using leaves collected from ash trees treated at an operational rate at the recommended application time (early summer) for emerald ash borer control and thus represented foliar concentrations from a realistic operational setting. The second experiment (Experiment 2) was conducted using leaves collected from a separate set of ash trees treated at the operational rate but in early autumn (September) to reduce the azadirachtin dissipation time and thus intentionally result in higher foliar test concentrations.

2.2. Microcosm design and deployment

Aquatic and terrestrial microcosms contained field-collected natural substrates (stream water, detritus, and wood pieces in aquatic microcosms, forest floor litter in terrestrial microcosms), and their description, deployment, and operation have been described previously (Kreutzweiser et al., 2007, 2008). Briefly, aquatic microcosms consisted of glass aquariums, 13 cm wide, 30 cm long, and 21 cm high, fitted with a Plexiglas lid. Each microcosm contained 6 L of stream water (collected from a forest stream at a single time), 300 mL of stream detritus (organic material collected from a forest stream, sieved to 1–5 mm particle sizes, frozen for 14 weeks to kill sediment organisms, then thawed for 5 days before being added to the microcosms), and 10 twigs from speckled alder (*Alnus incana* ssp. *rugosa*) trees (approximately 10 mm diameter and 15 cm long) to provide natural cover and sites of attachment for the test invertebrates. Water was not renewed over the 16-day experimental period, and water temperatures ranged 19–22 °C, pH was 7.3–7.6, specific conductance was 66–98 μS/cm, and dissolved oxygen was held near saturation (7.8–8.9 mg/L) by aeration.

Terrestrial microcosms were constructed of acrylic tubing, 7 cm diameter and 10 cm high, fitted with a plastic bottom containing two screened drainage holes, and covered on top with a metal lid containing four 3-mm diameter holes for air circulation. Each microcosm contained 60 g of field-collected litter from a hardwood forest, with the litter held at or corrected to ambient moisture by addition of de-ionized water just prior to being placed in the microcosms. The litter consisted of partially decomposed organic material (about 60% organic determined by ash-free dry mass) collected from under the recent leaf litter and above the mineral soil. The material was frozen for 12 weeks to kill litter invertebrates, and then thawed and held in open containers for 1 week before being added to the microcosms. Litter temperatures were not monitored, but air temperatures were 19–23 °C and relative humidity was 45–76% in the experimental laboratory that contained the microcosms. For both aquatic and terrestrial microcosm tests, daylight simulation fluorescent bulbs provided a 12 h light/12 h dark regime.

Representative decomposer invertebrates in aquatic microcosms were field-collected stonefly nymphs, *Pteronarcys dorsata*, and crane fly larvae, *Tipula* sp., tested together with 9 individuals of each taxon in each microcosm. Decomposer invertebrates in the terrestrial microcosms were litter-dwelling earthworms, *Eisenia fetida*, obtained from a commercial supplier (Cathy's Crawly Composters, Bradford, Ontario). The earthworms were tested in pairs with two clitellate (light-colored band present indicating sexual maturity) worms impartially allocated to each microcosm.

2.2.1. Experiment 1: leaves from operational field trial

Leaves were collected at senescence just before leaf-fall (early October) from azadirachtin-treated ash trees and from non-treated ash trees (to serve as controls). Azadirachtin-treated trees were stem-injected with TreeAzin™ by an EcoJect System® (BioForest Technologies Inc., Sault Ste Marie, Ontario) at the recommended operational rate of 0.2 g azadirachtin cm^{-1} diameter at breast height in early summer (end of June). The trees were on average 20 cm diameter and 9 m high. The leaves were sealed in plastic bags and held on ice in the dark for transfer to the laboratory where there were placed in a dark, 2 °C storage chamber before addition to the microcosms. The leaves were held in cold storage for 30 days for the aquatic microcosms and 45 days for the terrestrial microcosms. A previous study demonstrated that azadirachtin in leaf tissue was stable for at least three months under dark cold storage conditions (Grimalt et al., in press).

For aquatic microcosms, batches of leaves were collected from among 6 treated trees. The batches consisted of 24 individual leaflets collected from a specific area of a tree (i.e., collected from a few compound leaves in close proximity and at the same location within a tree), each batch collected, labeled and stored separately. This was intended to provide a range of azadirachtin concentrations among leaf batches, assuming differences in uptake among trees and within a tree. For terrestrial microcosms, the leaves were collected at the same time using the same approach but in smaller batches of 14 leaves. Leaves from two nearby non-treated ash trees were collected in a similar fashion to be used as controls.

Leaf batches from non-treated trees were added to five aquatic microcosms and five terrestrial microcosms to serve as controls. Leaf batches from treated trees were added to 20 aquatic microcosms, and 16 terrestrial microcosms, hereafter referred to as treated microcosms. Each aquatic microcosm received 12 whole leaves, held in three groups of four leaves by a plastic clip, and placed on the bottom substrates. Each also contained a plastic cup, screened on both ends with 0.5-mm mesh to exclude the aquatic insects, and containing 10 leaf disks (23-mm diameter) cut from additional leaves in the same batch for each microcosm. Leaf material in the plastic cup was used to measure mass loss by microbial decomposition. Each terrestrial microcosm received eight half-leaves (the other halves were analyzed for azadirachtin concentration) that were placed just below the surface of the litter. In a manner similar to the setup for the aquatic microcosms, a 0.5-mm mesh pack containing five leaf disks cut from additional leaves in the same batch was placed in the litter below the leaf pieces to measure mass loss by microbial decomposition.

2.2.2. Experiment 2: leaves from intentional high-dose trees

For the second experiment, leaves were collected at senescence just before leaf-fall (early October) from Tree-Azin™-treated ash trees that were injected in September and from non-treated trees that served as controls. The trees were on average 5 cm in diameter and three trees were stem-injected at a rate of 0.2 g azadirachtin cm^{-1} diameter at three sequential times, one week apart (1 tree each week) using the same EcoJect System®. This was to provide three trees

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across a gradient of dissipation times since treatment (and therefore a gradient of resulting foliar concentrations) to represent high (H=least time since treatment), medium (M), and low (L=greatest time since treatment) test concentrations. There were 7 replicate microcosms for each treatment group ($n=7$). The leaves were collected and held in a similar fashion to those from the operational trial (2.2.1). The aquatic and terrestrial microcosm protocols were also the same as those for the operational trial, except that whole leaves were used (rather than leaf disks) to measure aquatic and terrestrial microbial decomposition.

2.3. Azadirachtin test concentrations

Total azadirachtin concentrations (Aza-A+B) in leaf samples were measured using a liquid chromatography–mass spectrometery (LC–MS) technique as described by Grimalt et al. (in press). Briefly, the method involved accelerated solvent extraction of 0.5 g subsamples of the macerated leaf material. Acetonitrile extracts were cleaned up by passing the extract through a bed of primary secondary amine (0.1 g), evaporated to dryness under a stream of nitrogen, filtered (Acrodisc 0.20 μm) and reconstituted in 1 mL of HPLC grade acetonitrile : water 50:50 (v/v). A 25-μL aliquot of the final sample was injected into the LC–MS system and chromatographic separation of the Aza-A and Aza-B analytes was achieved using a reversed phase liquid chromatography (Kinetex C_{18} column, 50 mm × 2.1 mm i.d., 2.6 μm). Quantification was preformed using selected ions at m/z 743.2 and 685.2, respectively. A limit of detection at 0.01 mg kg^{-1} and quantification at 0.04 mg kg^{-1} was achieved for each analyte separately. Foliar concentrations were expressed as mg total azadirachtin (A+B) per kg of leaf fresh weight Confirmation of analyte identities were made based on additional diagnostic fragment ions. Validation studies showed average recovery efficiencies for Aza A and B of 91% and 84%, respectively, and precision ranging from 3% to 10% and 2% to 8% for the two analytes depending upon the fortification concentration level.

For terrestrial microcosms from the operational trial (Experiment 1), each leaf was cut in half immediately before addition to the microcosms and the batch of half leaves from each microcosm formed a composite sample for analysis of initial azadirachtin concentration. Samples were taken on the same day that the worms were added to the microcosms, and held frozen in the dark for subsequent analysis. This provided a measured average concentration of azadirachtin for each treated terrestrial microcosm and facilitated a comparison between control and treated microcosms and an examination of concentration-response trends.

For aquatic microcosms from Experiment 1, individual microcosm batch concentrations were not available (samples misplaced in transit). Rather, an average test concentration and a proportion of samples containing measurable azadirachtin concentrations were determined from a general subsample of 40 batches of three leaves each collected at the same time and place as those used in the actual treated microcosms. These batches of leaves were used to provide a surrogate measure of average test concentration among leaves added to the treated aquatic microcosms. Consequently, analyses among aquatic microcosms were restricted to comparisons between control and treated microcosms because actual test concentrations for individual microcosms were not available to determine concentration-response trends.

For terrestrial and aquatic microcosms in the intentional high-dose experiment (Experiment 2), a subset of 10 leaves was impartially drawn from each treatment batch (H, M, L) immediately before the leaves were added to the microcosms, and frozen for subsequent analysis. This provided a measured average test concentration for each treatment level.

2.4. Response measurements

2.4.1. Test organisms

The exposure periods for test organisms were 16 days in aquatic microcosms and 35 days in terrestrial microcosms. In terrestrial microcosms just prior to adding the earthworms, each pair was lightly rinsed with water to remove litter particles then weighed to determine initial pair-weights. At weekly intervals thereafter through the exposure period, the contents were removed and searched for living earthworms, and the worms were rinsed, re-weighed, and returned to the microcosms. Pair-weight measurements were discontinued when one or both of the worms in a microcosm were dead. At each weekly interval and at the end of the exposure period, the contents of each terrestrial microcosm were searched for cocoons to determine total cocoon production. At the end of the exposure period in aquatic microcosms, the bottom substrates were removed, searched for all insects, and the numbers of dead and alive individuals were recorded.

2.4.2. Leaf consumption

The amount of leaf material consumed by test organisms was determined by mass loss of leaf material. For mass loss determinations, leaves were taken from cold storage just before addition to the microcosms, air-dried for 2 h to stabilize fresh weights, then batch-weighed to provide initial fresh weights. Initial dry weights were estimated from a regression of dry weights on fresh weights of 50 ash leaves from the control trees (separate batches for aquatic and terrestrial microcosms, linear regression, $p<0.001$, $r^2=0.96$ for aquatic Experiment 1, $p<0.001$, $r^2=0.69$ for terrestrial Experiment 1, $p<0.001$, $r^2=0.90$ for aquatic Experiment 2, $p<0.001$, $r^2=0.86$ for terrestrial Experiment 2). The leaves were individually weighed, those for aquatic microcosms were pre-leached in running water for 24 h, dried at 60 °C to constant weight, then all leaves were re-weighed to determine initial dry weights and to account for leaching losses from leaves in aquatic microcosms. The leaves added to the microcosms were not initially dried and weighed to directly measure initial dry weights because the microcosm experiments were to simulate natural leaf-fall (fresh leaves added to microcosms), and to avoid the potential that drying the leaves could affect the palatability to test organisms and the stability of the azadirachtin. At the end of the experimental periods, remaining leaf material was removed, gently washed free of debris and biofilms, dried at 60 °C to constant weight and weighed. Total decomposition (mass loss) of leaf material from combined invertebrate feeding and microbial activity was determined as the difference between the estimated, initial batch dry weight of the leaves added to the microcosms, and the dry weight of leaf material remaining at the end of the experiment. Mass loss was expressed as the % lost from the initial weights.

2.4.3. Microbial decomposition rate

At the end of experimental periods in aquatic and terrestrial microcosms, leaf material was removed from the screened containers, washed gently to remove particles and biofilms without damaging the leaf material, dried at 60 °C to constant weight, then re-weighed to determine final dry weights. Mass loss by microbial decomposition was determined as the difference between estimated initial dry weights and measured final dry weights of the leaf disk batches, and expressed as % lost from initial weights. We recognize that the microbial contribution to total decomposition may not have been identical to microbial decomposition in these invertebrate-exclusion containers because of potential differences in microbial community structure or function in the presence/absence of macroinvertebrates, but could not account for this.

2.5. Data analysis

In Experiment 1 (leaves from operational field trial), differences in response measurements between control ($n=5$) and treated ($n=16$ terrestrial and $n=20$ aquatic) microcosms were tested by t-tests adjusted for unequal samples sizes, or by Mann–Whitney rank sum tests when equal variance tests failed. For terrestrial microcosms (for which test concentrations for each microcosm were available), relationships between azadirachtin concentrations and response variables were examined by scatter plots and linear regressions. In Experiment 2 (leaves from intentional high-dose trees), differences in response measurements among controls, low, medium, and high-dose treatments were determined by ANOVA (or Kruskal–Wallis test when equal variance failed), followed by Tukey's comparisons when the F-test was significant ($n=7$ terrestrial and 7 aquatic per treatment, $p<0.05$). All percent data were arcsine-square root transformed to improve normality.

3. Results

3.1. Experiment 1: leaves from operational field trial

3.1.1. Terrestrial microcosms

Of 57 composite leaf samples analyzed over the experimental period in terrestrial microcosms, 40 samples (70%) did not contain measureable concentrations of azadirachtin. Total azadirachtin concentrations among the remaining leaf samples ranged from 0.04 to 6.03 mg kg^{-1}, with a median of 0.17, mean of 0.65, and standard deviation of 1.43 mg kg^{-1}. Initial concentrations among leaves added to the 16 terrestrial microcosms ranged from non-detectable (8 of the 16) to 0.89 mg kg^{-1}, with a median of 0.29, mean of 0.38, and standard deviation of 0.33 mg kg^{-1}. The initial concentrations in leaves from each microcosm were taken as the test concentration for that microcosm. Overall, there were insufficient leaf samples from the terrestrial microcosms containing measureable concentrations to determine a dissipation rate in microcosms.

There was no mortality of earthworms in control or treated microcosms. There was no evidence of an adverse effect on feeding rates by earthworms in treated microcosms. The % mass loss of leaf material among all microcosms ranged from 31 to 78%, and the average mass loss by earthworm consumption was slightly but significantly greater in all treated microcosms than

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in controls (Table 1) (t-tests, $p=0.030$ and 0.004). There was no significant relationship between azadirachtin concentration and mass loss among all microcosms (regression, $r^2=0.12$, $p=0.112$), and although mass loss was weakly and positively associated with concentration among microcosms with leaves containing measurable azadirachtin ($r^2=0.33$, $p=0.024$), the mass loss at the highest test concentrations was within the range of mass loss among microcosms with little or no foliar azadirachtin (Fig. 1A).

There was no evidence of an adverse effect on earthworm growth rates over the 35-day experimental period. No significant difference in earthworm weight gain was detected between all treated microcosms and controls (t-test, $p=0.501$), and between microcosms with leaves containing measurable azadirachtin concentrations and controls (Mann–Whitney, $p=0.272$). There was a weak but positive relationship between concentration and earthworm weight gain among all microcosms ($r^2=0.13$, $p=0.060$) and among microcosms with leaves containing measurable azadirachtin ($r^2=0.53$, $p=0.024$) (Fig. 1B) indicating that earthworms grew slightly better in microcosms containing leaves from treated trees. Cocoon production varied widely among earthworm pairs, ranging from 1–14 cocoons pair^{-1}, but did not differ between microcosms with leaves from treated trees and those with leaves from controls (t-test, $p=0.702$). There was no relationship between cocoon production and foliar azadirachtin concentrations ($r^2<0.01$, $p=0.942$) (Fig. 1C).

The decomposition of leaf material by terrestrial microbial communities in microcosms was not inhibited by azadirachtin. Overall, mass loss by microbial decomposition activity in fine-mesh leaf packs ranged from 30–67%. There was no significant difference in microbial decomposition between all treated and control microcosms (t-test, $p=0.763$), nor between microcosms containing measurable azadirachtin and controls (t-test, $p=0.863$). There was no significant relationship between azadirachtin concentration and microbial decomposition among all microcosms ($r^2=0.07$, $p=0.253$), nor among microcosms with measurable azadirachtin ($r^2=0.08$, $p=0.359$) (Fig. 1 D).

Table 1
Median, mean, and standard error (SE) of leaf mass loss by earthworm consumption and microbial decomposition (total) and by microbial decomposition alone (microbial) in terrestrial microcosms of Experiment 1 containing leaves from non-treated trees (controls, $n=5$), leaves from all treated trees (treated, $n=16$), and the subset of microcosms with leaves containing measurable azadirachtin concentrations (measurable aza, $n=8$).

Treatment	Statistic	% Leaf mass loss (total)	% Leaf mass loss (microbial)
Control	Median	56.7	49.4
	Mean	50.7	50.6
	SE	4.2	2.5
Treated	Median	63.5	49.2
	Mean	63.9*	49.1
	SE	2.8	2.6
Measurable aza	Median	63.9	52.9
	Mean	66.1*	51.4
	SE	2.0	3.3

* Indicates significant difference ($p<0.05$) from control.

3.1.2. Aquatic microcosms

Similar to the leaf batches analyzed for terrestrial microcosms, leaves collected at senescence and added to aquatic microcosms contained little or no azadirachtin. Among 40 composite leaf samples analyzed over the 16-day experimental period in the auxiliary aquatic fate microcosms, 36 (90%) did not contain measureable concentrations of azadirachtin. In the remaining 4 leaf samples, concentrations ranged from 0.03 to 1.59 mg kg^{-1} with a median of 0.035, mean of 0.42, and standard deviation of 0.78 mg kg^{-1}.

Aquatic insect survival was not affected by exposure to leaves from treated trees. Among the stonefly *Pteronarcys*, there was no mortality in controls and a mean of 0.6% mortality (1 dead insect) among the 20 microcosms with treated leaves. Mortality of the cranefly *Tipula* was slightly higher but not related to consumption

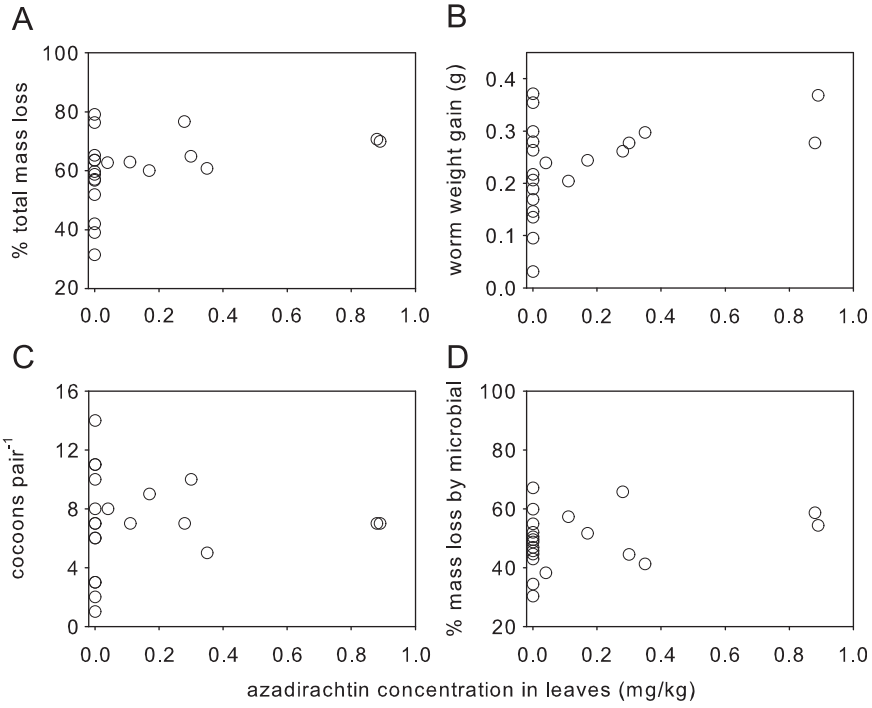

Fig. 1. Total leaf mass loss (A), earthworm weight gain (B), earthworm cocoon production (C), and leaf mass loss by microbial decomposition (D) in terrestrial microcosms of Experiment 1 containing leaves from control and azadirachtin-injected trees at the operational field rate.

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Table 2
Median, mean, and standard error (SE) of leaf mass loss by aquatic insect consumption and microbial decomposition (total) and by microbial decomposition alone (microbial) in aquatic microcosms of Experiment 1 containing leaves from controls ($n=5$), and leaves from all treated trees (treated, $n=20$).

Treatment	Statistic	% leaf mass loss (total)	% leaf mass loss (microbial)
Control	Median	13.4	0
	Mean	14.8	2.6
	SE	5.6	2.6
Treated	Median	41.7	13.8
	Mean	38.6*	14.9*
	SE	3.0	3.2

* Indicates significant difference ($p<0.05$) from control.

of treated leaves. There was an average of 4.4% mortality in controls and 2.8% mortality among treated microcosms.

Exposure to leaves from treated trees did not inhibit feeding by aquatic insects. The mass loss of leaf material in microcosms with treated leaves was significantly higher than in controls (t-test $p=0.001$) (Table 2). Part of this increased decomposition of treated leaves was attributable to breakdown by microbial communities as mass loss by microbial decomposition alone in fine-mesh leaf packs was significantly (7-fold) higher in microcosms containing leaves from treated trees than in controls (t-test $p=0.038$) (Table 2).

3.2. Experiment 2: leaves from intentional high-dose trees

3.2.1. Test concentrations

Azadirachtin concentrations in the batch samples (composite of 10 leaves) from the 3 high-dose trees were 0.91, 1.18, and 5.78 mg kg^{-1}, and these were taken as the low (L), medium (M), and high (H) test concentrations in terrestrial and aquatic microcosms.

3.2.2. Terrestrial microcosms

There was no mortality of earthworms by the end of the 35-day exposure period among control, low, or medium-dose microcosms, and 7% mortality (one dead earthworm) by day 35 in the high-dose microcosms. There was no indication that leaves from the high-dose trees inhibited feeding rates by earthworms. Most leaf material in the microcosms was consumed, and while the average total mass loss tended to be slightly higher in two of the treatment groups than in controls (Table 3), there was so significant difference among groups (Kruskall–Wallace, $p=0.878$). Leaf decomposition by microbial communities alone (fine-mesh packs) was significantly reduced at the highest concentration (H) (Tukey's, $p<0.05$), but was similar to controls at the medium and low concentrations (Table 3).

Although no significant differences in feeding rates were detected, earthworms in treated microcosms grew at a greater rate than those in controls, and the differences in weight gain were significant in the high (H) treatment (Table 4) (Tukey's $p<0.05$). Cocoon production overall was much lower in Experiment 2 than in Experiment 1 (Table 4) and no significant differences among treatments could be detected (ANOVA, $p=0.450$), although the low cocoon production precluded a rigorous assessment of effects on reproduction.

3.2.3. Aquatic microcosms

The survival of aquatic insects was not affected by exposure to treated leaves, even at the highest test concentration. Average mortality across treatments was $<10\%$ for both taxa (Table 5) and there were no significant differences among groups for *Pteronarcys* sp. (ANOVA $p=0.316$) or *Tipula* sp. ($p=0.258$). Feeding rates on leaf material by aquatic insects did not appear to be inhibited by exposure to leaves from the high-dose trees. Most

Table 3
Median, mean, and SE ($n=7$) of leaf mass loss by earthworm consumption and microbial decomposition (total) and by microbial decomposition alone (microbial), and weight gain by earthworms in terrestrial microcosms of Experiment 2 containing leaves from control and intentional high-dose trees.

Treatment	Statistic	% Leaf mass loss (total)	% Leaf mass loss (microbial)
Control	Median	89.5	54.6
	Mean	82.2	54.2
	SE	5.8	1.9
Low	Median	85.9	59.3
	Mean	85.9	60.5
	SE	2.1	3.1
Medium	Median	85.8	51.2
	Mean	82.4	51.1
	SE	3.9	2.8
High	Median	89.3	41.9
	Mean	83.0	43.9*
	SE	4.3	2.6

* Indicates significant difference ($p<0.05$) from control.

Table 4
Median, mean, and SE ($n=7$) weight gain and cocoon production by earthworms in terrestrial microcosms of Experiment 2 containing leaves from control and intentional high-dose trees.

Treatment	Statistic	Weight gain (g)	Number of cocoons $pair^{-1}$
Control	Median	0.194	1
	Mean	0.144	1.3
	SE	0.071	0.5
Low	Median	0.240	2
	Mean	0.285	1.4
	SE	0.059	0.4
Medium	Median	0.263	1
	Mean	0.261	0.7
	SE	0.063	0.3
High	Median	0.445	1
	Mean	0.407*	0.7
	SE	0.033	0.3

* Indicates significant difference ($p<0.05$) from control.

Table 5
Median, mean, and SE ($n=7$) of aquatic insect % mortality among treated and control microcosms of Experiment 2 containing leaves from control and intentional high-dose trees.

Treatment	Statistic	% Mortality *Pteronarcys* sp.	% Mortality *Tipula* sp.
Control	Median	0	0
	Mean	4.8	7.9
	SE	2.2	6.3
Low	Median	0	11
	Mean	6.3	7.9
	SE	3.3	3.2
Medium	Median	0	0
	Mean	1.6	0
	SE	1.6	0
High	Median	11	0
	Mean	6.3	6.3
	SE	2.2	3.3

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Table 6
Median, mean, and standard error (SE, $n=7$) of leaf mass loss by aquatic insect consumption and microbial decomposition (total) and by microbial decomposition alone (microbial) in aquatic microcosms of Experiment 2 containing leaves from control and intentional high-dose trees.

Treatment	Statistic	% Leaf mass loss (total)	% Leaf mass loss (microbial)
Control	Median	87.1	57.9
	Mean	86.8	61.2
	SE	0.6	2.9
Low	Median	89.1	70.8
	Mean	89.7	68.6
	SE	0.9	3.1
Medium	Median	84.1	67.8
	Mean	82.7*	66.7
	SE	1.4	2.6
High	Median	86.5	45.5
	Mean	85.9	47.5
	SE	0.89	7.2

* Indicates significant difference from control ($p<0.05$).

leaf material across all microcosms was consumed (average mass loss ranged 84–89%) and the total mass loss of leaves from the low- and high-concentration treatments was not significantly different from control (Tukey's, $p>0.05$). The average total mass loss in medium-concentration microcosms was slightly (about 4%) but significantly lower than in controls (Tukey's, $p<0.05$) (Table 6). Similar to terrestrial microcosms, the decomposition of leaf material in fine-mesh packs by aquatic microbial communities appeared to be reduced (14% less than controls) at the highest concentration, but a significant difference could not be detected (Tukey's, $p>0.05$). Mass loss by microbial decomposition was slightly, but not significantly, higher at the other two treatment levels than in controls (Table 6).

4. Discussion

Our field trials and microcosm experiments demonstrated that foliar azadirachtin concentrations resulting from systemic injections in trees are likely to pose little risk of harm to key decomposer organisms. When tree injections are made at the operationally recommended rate (0.2 g azadirachtin/cm) and time of year (early summer) for emerald ash borer control, azadirachtin concentrations in ash leaves will dissipate quickly, as indicated by the general lack of measurable concentrations in the leaves collected at senescence and added to the microcosms. This concurs with similar field trials from which McKenzie et al. (2010) and Grimalt et al. (in press) report 50% dissipation times for azadirachtin in tree foliage of about 5–22 days depending on the species and growing conditions. For example, mean azadirachtin foliar concentrations ranged from <0.01 to 0.09 mg kg^{-1} when sampled 70 days after injection, a time approximating the onset of leaf senescence, and were consistently below 0.05 mg kg^{-1} by 83 days post-treatment (Grimalt et al., in press). Therefore, it appears that most leaves will contain little or no measurable azadirachtin by the time of natural senescence and leaf fall in autumn. In our microcosms, leaves from the operational field trials did not induce significant, adverse effects on earthworms, aquatic insects, or microbial communities. Even when leaves from intentional high-dose trees were added, the only significant, adverse effect detected was a reduction in microbial decomposition of leaf material, and only at the highest test concentration ($\sim$6 mg kg^{-1}).

Based on a comparison between the present study and our previous assessment of imidacloprid, the results indicate that azadirachtin used as a systemic insecticide in ash trees for wood-borer control is likely to pose less risk of harm to decomposer organisms than when imidacloprid is used as a systemic insecticide. However, this lower risk to decomposer organisms is at least partly related to lower foliar concentrations of azadirachtin at senescence owing to inherently lower uptake and faster dissipation rates in tree foliage. In previous studies, we found that foliar imidacloprid concentrations at senescence in systemically treated ash trees were typically $\sim$1 mg kg^{-1}, and in systemically treated maple trees were 3–30 mg kg^{-1} with an average concentration of 8 and upper 95% confidence limit of 19 mg kg^{-1} (Kreutzweiser et al., 2008, 2009). In the present study, we showed that foliar azadirachtin concentrations in most leaf batches at senescence from ash trees were below the limit of detection (<0.01 mg kg^{-1}). When data from leaf batches used in both terrestrial and aquatic microcosms to measure initial concentrations (i.e., within 7 days of addition to microcosms) of the present study are combined, 65% of them were <0.01 mg kg^{-1} and the average of the 59 samples was 0.19 mg kg^{-1}. Therefore, since our results indicated no significant adverse effects on litter-dwelling earthworms or leaf-shredding aquatic insects at concentrations up to $\sim$6 mg kg^{-1}, this is a margin of safety of at least 30$\times$ the expected field concentrations (0.19 mg kg^{-1}) based on the average concentration of our 59 leaf samples added to microcosms. For adverse effects on microbial decomposition, this is a safety margin of at least 6$\times$ the expected field concentrations. Average foliar concentrations in ash trees by senescence in the samples reported by Grimalt et al. (in press) were <0.05 mg kg^{-1} and therefore at least 4-fold less than ours, indicating a broader margin of safety. Our higher average concentration resulted from a couple of leaf samples with unexpectedly high concentrations (1.6 and 6.0 mg kg^{-1}) out of 59 samples. In previous studies, we detected sublethal feeding-inhibition effects on earthworms and aquatic insects at foliar imidacloprid concentrations of $\sim$1–3 mg kg^{-1} (Kreutzweiser et al., 2007, 2008) but here we found no such effects at foliar azadirachtin concentrations up to 5.8 mg kg^{-1}. Thus by comparison of our previous and current studies in terms of expected foliar concentrations at senescence and their non-target effects, we infer that azadirachtin will pose less risk of harm to decomposer invertebrates than imidacloprid at operational field application rates. However, a direct side-by-side comparative study of the two systemic insecticides in the same experimental setting would provide a more rigorous relative assessment.

While no significant, adverse effects (i.e., reductions) other than reduced microbial decomposition at the highest test concentration were detected, significant increases in some response variables were measured and these could be construed as insecticide impacts. The total mass loss of leaf material (combined invertebrate consumption and microbial decomposition) tended to be higher in terrestrial and aquatic microcosms with treated leaves than in controls. At least part of the increased total mass loss of treated leaves was attributable to increased microbial decomposition, as mass loss of treated leaves in fine-mesh bags was usually higher (sometimes significantly higher) in all but the highest test concentration among treated terrestrial and aquatic microcosms than in controls. Accelerated microbial growth and activity at low levels of some other insecticides in aquatic and soil environments have been noted previously (Lopez et al., 2002; Vig et al., 2008) and often reflects microbial mineralization of the insecticide as a source of nutrients (Karpouzas and Singh, 2008). However, at the highest foliar azadirachtin concentration (5.8 mg kg^{-1}), microbial decomposition of treated leaf material was clearly reduced in comparison to controls and other treatment levels. The threshold for an adverse effect of azadirachtin on microbial communities is evidently between 1.2 and 5.8 mg kg^{-1} total foliar azadirachtin. Significantly higher growth of earthworms

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at the highest test concentration may have reflected the increased availability of litter biomass available for consumption owing to reduced microbial decomposition of leaf material at that concentration.

It is possible that the 16-day exposure period in aquatic microcosms was insufficient to detect an adverse effect of azadirachtin on aquatic insects. Water in the microcosms was not renewed over the 16 days and the experiments were ended at that time to avoid an accumulation of metabolic wastes and because the breakdown of organic matter significantly increased turbidity by the end of the experimental period making it impossible to see the insects or the residual leaf material. Azadirachtin is known to exhibit strong antifeedent, growth regulation and reproductive effects among target invertebrates (Schmutterer, 1990; Isman, 1997), and the exposure period may not have been long enough to invoke antifeedent or growth-regulation (molt inhibiting) effects on the insects. However, tests with azadirachtin against several insect pest species have shown high mortality from growth-regulatory and (or) antifeedent effects within 1–14 days of exposure (Helson et al., 2001; Kumar and Poehling, 2006; Schulte et al., 2006; Seljasen and Meadow, 2006). Similarly, in previous experiments with non-target aquatic insects exposed to aqueous azadirachtin concentrations (Kreutzweiser et al., 1999, 2000; El-Shazly and El-Sharnoubi, 2000), significant reductions in survival were detected within 5–9 days of exposure, although the rapid non-target effects were detected only at concentrations well above expected field concentrations. This suggests that the 16-day exposure periods in aquatic microcosms should have been sufficient to exhibit significant mortality or feeding inhibition in aquatic insects if the foliar azadirachtin concentrations had induced such effects. Nevertheless, further tests with realistic foliar azadirachtin concentrations against leaf-shredding aquatic insects over longer periods and through at least one molting cycle would be advisable.

We could find no other published studies reporting non-target effects of foliar azadirachtin concentrations with which to compare our results. Previous studies into effects of azadirachtin as a systemic insecticide have focused on pest (target) invertebrates. However, a number of studies have shown that when azadirachtin is used as a topical or foliar pesticide, it generally has a favorable environmental toxicology profile showing relatively low toxicity and few or limited non-target effects on mammals, birds, beneficial arthropods, fish, and aquatic invertebrates at field-expected concentrations (Schmutterer, 1990; Stark, 2007; Thompson and Kreutzweiser, 2007). At least one notable exception is the high, selective toxicity to one group of aquatic microcrustaceans, adult copepods, at well below expected concentrations in standing water bodies (Kreutzweiser et al., 2004), but this is not relevant to leaf-shredding insects exposed to foliar concentrations. Thus the lack of significant adverse effects on key decomposer organisms at realistic foliar concentrations in the present study are in line with most previous studies showing limited or non-detectable effects of azadirachtin on beneficial invertebrates.

5. Conclusions

The use of azadirachtin as a systemic insecticide for control of wood-boring insect pests such as emerald ash borer is unlikely to pose a significant risk of harm to aquatic or terrestrial decomposer invertebrates when leaves fall from insecticide-treated trees. This conclusion is based on a large margin of safety between average measured concentrations in senescent leaves (most of which were below the limit of detection of 0.01 mg kg^{-1}) and the threshold for adverse effects at 5.8 mg kg^{-1} or above for most toxicity measurement endpoints. We therefore suggest that azadirachtin could be a preferred option for a systemic insecticide to control wood-boring insect pests, especially in ecologically sensitive environments such as riparian (shoreline) and source-water forests, wooded wetlands, or conservation areas.

We can identify at least two outstanding issues pertinent to the assessment of risk to aquatic decomposer invertebrates from foliar concentrations of azadirachtin that warrant further study. As noted earlier and given the known molt-inhibiting properties of azadirachtin, longer exposure periods for leaf-shredding insects in aquatic microcosms may be necessary to assess potential effects on molting success and subsequent survival. Secondly, given the high, selective toxicity of azadirachtin to some aquatic micro-crustaceans (Kreutzweiser et al., 2004), similar experiments to determine effects of foliar azadirachtin on key leaf-shredding macro-crustaceans, such as freshwater amphipods, would be advisable.

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References

Ascher, K.R.S., 1993. Nonconventional insecticidal effects of pesticides available from the neem tree, *Azadirachta indica*. Arch. Insect Biochem. Physiol. 22, 433–449.

Boeke, S.J., Boersma, M.G., Alink, G.M., van Loon, J.J.A., van Huis, A., Dicke, M., Rietjens, I.M., 2004. Safety evaluation of neem (*Azadirachta indica*) derived pesticides. J. Ethnopharmacol. 94, 25–41.

Cappaert, D., McCullough, D.G., Poland, T.M., Siegert, N.W., 2005. Emerald ash borer in North America: a research and regulatory challenge. Am. Entomol. 51, 152–165.

Ellison, A.M., Bank, M.S., Clinton, B.D., Colburn, E.A., Elliott, K., Ford, C.R., Foster, D.R., Kloeppel, B.D., Knoepp, J.D., Lovett, G.M., Mohan, J., Orwig, D.A., Rodenhouse, N.L., Sobczak, W.V., Stinson, K.A., Stone, J.K., Swan, C.M., Thompson, J., Von Holle, B., Webster, J.R., 2005. Loss of foundation species: consequences for the structure and dynamics of forested ecosystems. Front. Ecol. Environ. 3, 479–486.

El-Shazly, M.M., El-Sharnoubi, E.D., 2000. Toxicity of a neem (*Azadirachta indica*) insecticide to certain aquatic organisms. J. Egypt. Soc. Parasitol. 30, 221–231.

Gandhi, K.J.K., Herms, D.A., 2010. North American arthropods at risk due to widespread *Fraxinus* mortality caused by the alien emerald ash borer. Biol. Invasions 12, 1839–1846.

Graça, M.A.S., 2001. The role of invertebrates on leaf litter decomposition in streams—a review. Int. J. Hydrobiol. 86, 383–393.

Grimalt, S., Thompson, D., Chartrand, D., McFarlane, J., Helson, B., Lyons, B., Meating, J., Scarr, T., in press. Foliar residue dynamics of azadirachtins following direct stem injection into white and green ash trees for control of emerald ash borer. Pest Manage. Sci. doi:10.1002/ps.2183.

Hattenschwiler, S., Tiunov, A.V., Scheu, S., 2005. Biodiversity and litter decomposition in terrestrial ecosystems. Annu. Rev. Ecol. Evol. Syst. 36, 191–218.

Helson, B.V., Lyons, D.B., Wanner, K.W., Scarr, T.A., 2001. Control of conifer defoliators with neem-based bioinsecticides using a novel injection device. Can. Entomol. 133, 729–744.

Isman, M.B., 1997. Neem insecticides. Pestic. Outlook 8, 32–38.

Karpouzas, D.G., Singh, B.K., 2008. Microbial degradation of organophosphorus xenobiotics: metabolic pathways and molecular basis. Adv. Micro. Physiol. 51, 119–185.

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Kovacs, K.F., Haight, R.G., McCullough, D.G., Mercader, R.J., Siegert, N.W., Liebhold, A.M., 2010. Cost of potential emerald ash borer damage in U.S. communities, 2009-2019. Ecol. Econ. 69, 569–578.

Kreutzweiser, D.P., Capell, S.S., Scarr, T.A., 1999. Acute lethal and sublethal effects of a neem-based insecticide on nontarget aquatic insects in stream channels. Bull. Environ. Contam. Toxicol. 63, 365–371.

Kreutzweiser, D.P., Capell, S.S., Scarr, T.A., 2000. Community-level responses by stream insects to neem products containing azadirachtin. Environ. Toxicol. Chem. 19, 855–861.

Kreutzweiser, D.P., Sutton, T.M., Back, R.C., Pangle, K.L., Thompson, D.G., 2004. Some ecological implications of a neem (azadirachtin) insecticide disturbance to zooplankton communities in forest pond enclosures. Aquat. Toxicol. 67, 239–254.

Kreutzweiser, D.P., Good, K.P., Chartrand, D., Scarr, T.A., Thompson, D.G., 2007. Non-target effects on aquatic decomposer organisms of imidacloprid as a systemic insecticide to control emerald ash borer in riparian trees. Ecotoxicol. Environ. Saf. 68, 315–325.

Kreutzweiser, D.P., Good, K.P., Chartrand, D.T., Scarr, T.A., Thompson, D.G., 2008. Are leaves that fall from imidacloprid-treated maple trees to control Asian longhorned beetles toxic to non-target decomposer organisms? J. Environ. Qual. 37, 639–646.

Kreutzweiser, D.P., Thompson, D.G., Scarr, T.A., 2009. Imidacloprid in leaves from systemically-treated trees may inhibit litter breakdown by non-target invertebrates. Ecotoxicol. Environ. Saf. 72, 1053–1057.

Kumar, P., Poehling, H-M., 2006. Persistence of soil and foliar azadirachtin treatments to control sweetpotatoe whitefly *Bemisia tabaci* (Homoptera: Aleyrodidae) on tomatoes under controlled (laboratory) and field (netted greenhouse) conditions in the humid tropics. J. Pestic. Sci. 79, 189–199.

Lopez, L., Pozo, C., Gomez, M.A., Calvo, C., Lopez, J.G., 2002. Studies on the effects of the insecticide aldrin on aquatic microbial populations. Int. Biodeterior. Biodegrad. 50, 83–87.

McKenzie, N., Helson, B., Thompson, D., Otis, C., McFarlane, J., Buscarini, T., Meating, J., 2010. Azadirachtin: an effective systemic insecticide fro control of *Agrilus planipennis* (Coleoptera: Buprestidae). J. Econ. Entomol. 103, 708–717.

Mercader, R.J., Siegert, N.W., Liebhold, A.M., McCullough, D.G., 2011. Simulating the effectiveness of three potential management options to slow the spread of emerald ash borer (*Agrilus planipennis*) populations in localized outlier sites. Can. J. For. Res 41, 254–264.

Poland, T.M., McCullough, D.G., 2006. Emerald ash borer: invasion of the urban forest and threat to North America's ash resource. J. For. 104, 118–124.

Poland, T.M., Haack, R.A., Petrice, T.R., Miller, D.L., Baurer, L.S., Gao, R., 2006. Field evaluations of systemic insecticides for control of *Anoplophora glabripennis* (Coleoptera: Cerambycidae) in China. J. Econom. Entomol. 99, 383–392.

Schmutterer, H., 1990. Properties and potential of natural pesticides from the neem tree, Azadirachta indica. Ann. Rev. Entomol. 35, 271–297.

Schulte, M.J., Martin, K., Sauerborn, J., 2006. Effects of azadirachtin injection in litchi trees (*Litchi chinensis* Sonn.) on the litchi stink bug (*Tessaratoma papillosa* Drury) in northern Thailand. J. Pest. Sci. 79, 241–250.

Seljasen, R., Meadow, R., 2006. Effects of neem on oviposition and egg and larval development of *Mamestra brassicae* L: dose response, residual activity, repellent effect and systemic activity in cabbage plants. Crop Protect. 25, 338–345.

Smitley, D.R., Rebek, E.J., Royalty, R.N., Davis, T.W., Newhouse, K.F., 2010. Protection of individual ash trees from emerald ash borer (Coleoptera: Buprestidae) with basal soil applications of imidacloprid. J. Econom. Entomol. 103, 119–126.

Stark, J.D., 2007. Ecotoxicology of Neem. ACS Symposium Series 947, pp. 275–286.

Thompson, D.G., Kreutzweiser, D.P., 2007. A review of the environmental fate and effects of natural "reduced-risk" pesticides in Canada. In: Racke, K.D., A Felsot, A. (Eds.), Crop Protection Products for Organic Agriculture: Environmental, Health, and Efficacy Assessment. ACS Books, American Chemical Society, Washington, pp. 245–274.

Vig, K., Singh, D.K., Agarwal, H.C., Dhawan, A.K., Dureja, P., 2008. Soil microorganisms in cotton fields sequentially treated with insecticides. Ecotoxicol. Environ. Saf. 69, 263–276.

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