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Acute Lethal and Sublethal Effects of a Neem-Based Insecticide on Nontarget Aquatic Insects in Stream Channels

D. P. Kreutzweiser,[1] S. S. Capell,[1] T. A. Scarr[2]

[1] Canadian Forest Service, Great Lakes Forestry Centre, 1219 Queen Street East, Sault Ste. Marie, Ontario, Canada P6A 5M7
[2] Ontario Ministry of Natural Resources, Forest Resources Group, Suite 400, 70 Foster Drive, Sault Ste. Marie, Ontario, Canada P6A 6V5

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Extracts from seeds and leaves of the neem tree, *Azadirachta indica*, express insecticidal activity against a broad range of insect pests (Schmutterer and Singh 1995). The primary active ingredient in neem extracts is the triterpenoid, azadirachtin. Because several common forest pests are among the most susceptible insects to azadirachtin, the use of neem-based insecticides in Canadian forest pest management programs is being investigated (Isman et al. 1991; Helson et al. 1999). Insecticide applications to forests may result in some of the product entering nearby water bodies and it is therefore important to ensure that these insecticides do not post a risk of adverse effects on aquatic organisms. Small streams are particularly susceptible to contamination by runoff from adjacent sprayed areas because of low dilution potential, and from inadvertent overspray because they are difficult to avoid during aerial applications. We investigated the potential for adverse effects on stream insects by applying a neem-based insecticide to outdoor stream channels and measuring acute lethal and sublethal responses.

In a Canadian forest pest management context (application rate of 50 g/ha azadirachtin) the expected environmental concentration (EEC) in receiving waters is 0.035 mg/L azadirachtin. In previous laboratory tests, field-collected aquatic insects were unaffected by neem at or near the azadirachtin EEC (Kreutzweiser 1997). Scott and Kaushik (1998) also exposed non-target aquatic invertebrates to a neem insecticide in laboratory tests and concluded that field applications were unlikely to result in significant adverse effects. In contrast, Dunkel and Richards (1998) conducted laboratory bioassays against nontarget stream insects and reported 24-hr median lethal concentrations equivalent to azadirachtin concentrations close to the EEC. In the present study, we evaluated the risk of harm to stream insects under more realistic conditions, i.e., outdoor stream channels and short-term (5hr) exposures.

MATERIALS AND METHODS

Experiments were conducted at the Icewater Creek Research Area, 50 km north of Sault Ste. Marie, Ontario, Canada. Aquatic insects were collected from nearby forest streams, transported to the test site and held for 24 hr before testing in screened, floating cages in Icewater Creek adjacent to the stream channels. Kreutzweiser and Capell (1992) describe the stream channel configuration and test

Correspondence to: D. P. Kreutzweiser

procedures. Briefly, water was diverted from Icewater Creek, a third-order forest stream, through PVC channels measuring 30 cm wide by 7 m long and was treated with the test material at the midpoints of the channels. The upper (control) and lower (treated) sections of the channels each contained a test unit in which invertebrate response was monitored. The tests units were constructed of Plexiglas® and were 70 cm long, 30 cm wide, 10 cm high, screened on both ends with 1-mm mesh stainless steel, had removable lids, and were longitudinally divided into three lanes. A 35-cm section of each lane contained natural stream bottom substrate where test organisms were placed prior to the insecticide applications. Insects that dislodged from the substrate in each lane were observed drifting through the next 25-cm section (no substrate) and into the lower end (collector) of the lane. The floor of the collectors was recessed to allow the invertebrates to escape the current and each collector contained a stone to provide a site for reattachment.

The insecticide was applied to the mid section of the channels from stainless steel pyramid shaped applicators designed to deliver an exponentially declining test concentration over a 5-hr exposure period. The concentration profiles delivered by these applicators (Figure 1) are typical of concentration profiles in stream water measured after aerial applications of pesticides (Sundaram 1991; Kreutzweiser and Wood 1991; Thompson et al. 1991). The test material applied to the stream channels was the commercial neem formulation, Neemix® 4.5 (Thermo Trilogy Corporation, Columbia, MD), at a maximum test concentration of 1.05 mg/L azadirachtin (30 X the EEC). When a significant response was detected, the species was re-tested at 0.35 mg/L (10 X the EEC). Water samples were collected at 12 random times from the outlets of the channel lanes to check for the accuracy of test concentrations. These were frozen within 6 hr of collection and subsequently analyzed by HPLC (Sundararn et al. 1995).

During the 5-hr applications, observers recorded insect behavioral responses (those that dislodged from the substrate and drifted into the collectors) and the total number of drifted insects in treated units was compared to the natural drift rate in the control units over the 5-hr application period. The collectors were checked daily for dead insects and at the end of the 5-d observation period the substrate from each lane was searched, and the total number of alive and dead individuals was recorded. Differences between replicate treated and control lanes in insect drift and mortality were determined by randomization tests of G statistics for independence derived from 3-way contingency tables (response X treatment X replicate) (Pitt and Kreutzweiser 1998). A significant difference ($p<0.05$) in response measurements between treated and control units was considered a significant treatment effect.

For each test, 15 specimens of one taxon were placed in each of the three replicate lanes of a test unit, for a total of 45 individuals exposed to the treatment and response was compared to 45 individuals in replicate lanes of the control unit. In all, 9 separate taxa were tested in sequential experiments. These included the Plecoptera (stoneflies) *Isogenoides* sp., *Acroneuria* sp., and *Pteronarcys dorsata*,

the Ephemeroptera (mayflies) *Heptagenia flavescens*, and *Isonychia bicolor/rufa*, the Trichoptera (caddisflies) *Hydropsyche bifida/recurvata, Oligostomis pardalis,* and *Pycnopsyche guttifer*, and the Odonata (dragonflies) *Ophiogomphus* sp. For most taxa, both the short-term sublethal responses (drift over 5hr) and the longer-term survival rates (5d after treatment) were monitored. The 5-d survival of the mayfly *H. flavescens* after exposure to the insecticide was not evaluated because of high mortality (about 50%) in the control units by day 5. The sublethal drift response by the caddisfly *O. pardalis* was not determined because the individuals in the test lanes did not settle into the substrate portions during the pre-treatment period.

RESULTS AND DISCUSSION

The 12 random "spot checks" of azadirachtin concentrations indicated that actual test concentrations tended to be lower than nominal concentrations. The target concentrations for a given time during the 5-hr exposure were taken from the concentration profile (Figure 1) and compared to the measured concentrations in samples collected at the given time. The measured concentrations were approximately 80% of nominal concentrations (n=12, mean = 80.2%, S.E.= 4.9). Because the samples were collected from the channel outflows, this may represent loss of azadirachtin through adsorption to the natural substrate and channel walls. Although it is difficult to determine exact concentrations to which the test insects were exposed in the channels, the test concentrations have been corrected to 80% of nominal in the results given below.

Among eight taxa tested for behavioral (drift) responses to Neemix at the maximum test concentration of 0.84 mg/L azadirachtin, only the stonefly, *Isogenoides* sp., exhibited a drift rate (64%) that was significantly different from controls (Table 1). At the lower test concentration of 0.28 mg/L, the mean drift rate of *Isogenoides* sp. was 6.7% and this was not significantly different from controls ($p=0.693$). This demonstrates that there were no detectable avoidance responses to Neemix by most aquatic insects tested at concentrations well above the EEC of 0.035 mg/L.

The survival of three taxa among eight tested was negatively affected by exposure to the maximum test concentration of 0.84 mg/L azadirachtin. The species that demonstrated significant drift response at this concentration, *Isogenoides* sp., also incurred 40% mortality by the end of the observation period, and this was significantly different ($p=0.026$) from the relatively high natural mortality (22%) in control units (Table 2). Mortality was also significantly higher among channels treated at 0.84 mg/L than in controls for the mayfly, *Isonychia bicolor/rufa* ($p<0.001$), and the caddisfly, *Hydropsyche bifida/recurvata* ($p<0.001$). *Isogenoides* sp. was not re-tested at a lower concentration, but there were no significant differences in mortality between controls and channels treated at 0.28 mg/L azadirachtin for *I. bicolor/rufa* ($p=0.201$) or *H. bifida/recurvata* ($p=0.135$).

These experiments have demonstrated that aquatic insects typical of those found in

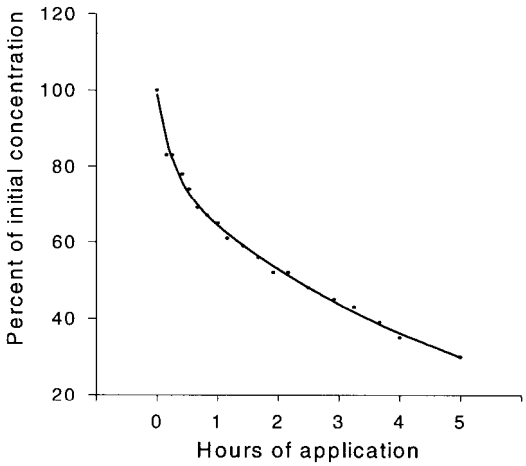

Figure 1. Concentration profile (as % of initial concentration) of a tracer dye applied to stream channels with pyramid-shaped applicators.

Table 1. Drift of aquatic insects in streams channels (n=3, 15 individuals per replicate) at the end of the 5-hr exposure period.

Taxa	Concentration (mg/L)[a]	Mean % drift (± 1 SE)	G-test[b] probability
Isogenoides sp.	0	0	
(stonefly)	0.28	6.7 (±3.8)	p=0.693
	0.84	64.4 (±8.0)	p=0.009
Acroneuria sp.	0	11.1 (±5.8)	
(stonefly)	0.84	11.1 (±4.4)	p=0.393
Pteronarcys dorsata	0	0	
(stonefly)	0.84	4.4 (±4.4)	n.a.[c]
Heptagenia flavescens	0	8.9 (±2.2)	
(mayfly)	0.84	4.4 (±2.2)	p=0.402
Isonychia bicolor/rufa	0	6.7 (±6.7)	
(mayfly)	0.84	6.7 (±3.8)	p=0.191
Hydropsyche bifida/recurvata	0	0	
(caddisfly)	0.84	2.2 (±2.2)	n.a.
Pycnopsyche guttifer	0	0	
(caddisfly)	0.84	0	n.a.
Ophiogomphus sp.	0	26.7 (±10.2)	
(dragonfly)	0.84	15.5 (±4.4)	p=0.065

[a] Concentrations are mg/L azadirachtin.

[b] The p values are from randomization tests using a G statistic for independence, control vs treated.

[c] Frequency of response insufficient for analysis.

small forest streams are not particularly sensitive to azadirachtin as applied in the Neemix formulation. This suggests that there is little risk of direct negative effects on aquatic invertebrates resulting from contamination of streams by aerial applications of Neemix® 4.5 in a forest pest management context. The concentration at which no significant effects were detected (0.28 mg/L) is about 8 X the EEC in a forest use scenario. The EEC (0.035 mg/L) was calculated as the concentration in 15 cm of water resulting from direct overspray at the allowable application rate, and is the approach used by the Canadian Pest Management Regulatory Agency for hazard evaluation of pesticide exposure in aquatic systems (Anonymous 1993). From studies evaluating the effectiveness of neem formulations as forest pest control agents, it appears that an application rate of 50 g/ha azadirachtin will provide sufficient control of several insect pests (Helson et al. 1999).

Table 2. Mortality of aquatic insects in stream channels (n=3, 15 individuals per replicate) at the end of a 5-d observation period.

Taxa	Concentration (mg/L)[a]	Mean % mortality (± 1 SE)	G-test[b] probability
Isogenoides sp.	0	22.2 (±4.4)	
(stonefly)	0.84	40.0 (±3.8)	p=0.026
Acroneuria sp.	0	11.1 (±5.9)	
(stonefly)	0.84	11.1 (±4.4)	p=0.134
Pteronarcys dorsata	0	2.2 (±2.2)	
(stonefly)	0.84	6.7 (±3.8)	p=0.198
Isonychia bicolor/rufa	0	4.4 (±2.2)	
(mayfly)	0.28	13.3 (±3.8)	p=0.201
	0	4.4 (±2.2)	
	0.84	28.9 (±2.2)	p<0.001
Hydropsyche bifida/recurvata	0	13.3 (±7.7)	
(caddisfly)	0.28	11.1 (±4.4)	p=0.135
	0	11.1 (±4.4)	
	0.84	28.9 (±5.8)	p<0.001
Oligostomis pardalis	0	4.4 (±4.4)	
(caddisfly)	0.84	11.1 (±2.2)	p=0.204
Pycnopsyche guttifer	0	0	
(caddisfly)	0.84	0	n.a.[c]
Ophiogomphus sp.	0	8.8 (±8.8)	
(dragonfly)	0.84	6.7 (±0)	p=0.598

[a] Concentrations are mg/L azadirachtin.

[b] The p values are from randomization tests using a G-statistic for independence, control vs treated.

[c] Frequency of response insufficient for analysis.

The results from this study concur with previous laboratory-based experiments which indicated that the use of neem-based insecticides in field applications should not pose a risk of direct harmful effects on non-target aquatic invertebrates (Kreutzweiser 1997; Scott and Kaushik 1998). The results do not support the

contention by Dunkel and Richards (1998) that azadirachtin formulations are toxic to stream-dwelling insects and could pose a risk of harm under operational conditions. This discrepancy may be due to differences among test species, test protocols, exposure duration, and in particular, differences among formulations of neem insecticides. As indicated by Dunkel and Richards (1998), the "inert" ingredients of neem formulations may significantly influence the toxicity of neem products to non-target aquatic insects. Their bioassays were conducted with the Align® formulation (Agridyne, Salt Lake City, UT). The present study focused on the Neemix® 4.5 formulation because it is being evaluated as a potential forest insecticide in Canada. It is currently registered as an agricultural insecticide in the USA (EPA Reg. No. 70051-9).

Given that the experiments of the present study were conducted under more natural conditions (outdoor stream channels, realistic exposure regimes) than the previous laboratory tests cited above, these results may provide a better assessment of the potential for adverse effects on stream insects. The risk of these effects appears small when azadirachtin is applied as the Neemix® 4.5 formulation under a forest use scenario. Further studies with a larger array of representative test species or with community-level experiments would improve predictive capabilities.

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