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Fate and Persistence of Azadirachtin A Following Applications to Mesocosms in a Small Forest Lake

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Azadirachtin, a natural product derived from the neem tree (*Azadirachta indica)* has proven to be effective against a broad range of insect pests (Rembold 1989; Schmutterer 1990; Hansen et al. 1994), including several of importance in Canadian forest pest management (Isman et al. 1990; Helson et al. 1999). The commercial formulation Neemix® 4.5 (® Thermo Trilogy Corp., Columbia, MD), has recently been granted temporary registration for aerial applications against sawfly pests in Canadian forestry (PMRA 2000). In the regulatory decision document, concerns were raised regarding potential effects on fish and zooplankton species which are considered most sensitive to azadirachtin intoxication (PMRA 2000). As a measure mitigating against potential deleterious effects in aquatic ecosystems, protective 50 m buffer zones were stipulated as a registration requirement.

The potential for deleterious effects of pesticides on aquatic organisms is a function of both toxicity and potential exposure. Exposures, in turn are determined largely by fundamental physico-chemical properties of the product. Azadirachtin is poorly to moderately soluble in water (0.05 mg/L), has negligible potential for bioconcentration or bioaccumulation in organisms (log Kow = 1.9), and is susceptible to photodegradation ($\lambda_{max} \sim 220$ nm) (PMRA 2000). Relatively few studies have investigated the fate and behaviour of azadirachtin in aquatic systems under natural conditions pertinent to Canadian forestry. Aquatic fate and dissipation studies which are available suggest that azadirachtin isomer A (AZA-A) is susceptible to both base-catalyzed hydrolysis and photolysis, with microbial degradation playing a relatively minor role (Sundaram 1996).

As part of an ongoing environmental assessment program focused on azadirachtin, we examined the fate and environmental effects of Neemix 4.5 using in-situ enclosures or mesocosms deployed in a small forest lake. In this paper we describe results pertaining to the fate and persistence of AZA-A in the water column including residues in both the dissolved phase and sorbed to suspended sediments. Detailed results describing effects on the zooplankton community are published elsewhere (Kreutzweiser et al. 2001).

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MATERIALS AND METHODS

In-situ enclosures or mesocosms as described previously (Thompson et al. 1992), were deployed in small lake (surface area approximately 150 m x 200 m) located 65 km northeast of Sault Ste. Marie, Ontario (latitude 46° 53' 25", longitude 84° 07' 76"). The lake is typical of those most susceptible to contamination via accidental overspray or drift during aerial pesticide applications for forest insect pest control. The site is characterized by highly organic bottom substrate, with submergent and floating aquatic macrophytes comprised mainly of bladderwort (*Utricularia* spp.) and pondweed (*Potamogeton* spp.). Seasonal average values for water chemistry parameters, as determined from replicate (N=5) samples taken from control mesocosms, were: pH = 5.3-5.9, conductivity = 12-16 µS/cm, alkalinity = 0.01-0.03 meq/L HCO_3^-, dissolved organic carbon (DOC) concentrations = 4.5-6.0 mg/L, total suspended particulate matter = 0.5-3.5 µg/L, total N = 0.26-0.38 mg/L and total P = 0.004-0.006 mg/L. Mid-day water temperatures ranged from 6-23°C. Water quality data were obtained using a Hydrolab® Minisonde Surveyor 4 water quality analyzer (pH, conductivity, temperature) or via standard chemical analyses of 100 mL filtered water samples for total N, total P, dissolved organic carbon, and major ion concentrations (Biogeochemistry Anaytical Laboratory at the Great Lakes Forestry Centre).

The commercial product, Neemix® 4.5, was applied to the surface of each treated mesocosm using a CO_2-powered backpack sprayer (model 4F, R&D Sprayers Inc., Opelousas, LA). A polyethylene curtain was used to isolate each enclosure during chemical applications and thereby prevent any potential for cross-contamination. Two experiments were conducted to allow assessment of temporal variation in aquatic fate and persistence. For the first experiment, applications were made between 0730 and 1000 h on 15 June 1999, to yield concentrations ranging from the expected environmental concentration (EEC) as calculated by the PMRA (1993) to 50 X the EEC. Nominal and observed concentrations are provided in Table 1. Details of the chemical application procedure are provided in Kreutzweiser et al. (2001). To verify initial chemical concentrations, chemical residue samples were taken within 4 h of treatment for all mesocosms. Thereafter, to assess the fate and persistence of AZA-A over time, chemical residue samples were collected from replicate (N=5) mesocosms treated at nominal levels of 0.035 and 0.70 mg/L only. In the first experiment, fate and persistence sampling involved 13 discrete sampling events over a 50-day period of observation. In the second experiment, applications were made on July 6, 1999 and sampling was restricted to the medium concentration only with 7 discrete sampling events over a 29-day period. All chemical residue samples were collected using a depth integrating 3-cm diameter aluminum tube with a foot valve. Composite raw water samples, comprised of nine subsamples from each mesocosm, were pooled in amber-coloured 4-L glass Winchester bottles.

In the laboratory, bottles were vigorously shaken to ensure homogeneity. A 2 L portion of each raw water sample was filtered through pre-weighed filters (1 µm

pore size, Whatman glass fibre filter + Whatman # 1; Whatman International Ltd., Maidstone, England) to remove suspended organic matter. Suspended organic matter retained on the filters were air-dried, weighed and placed in ziploc bags. A 1-litre aliquot of the filtrate was transferred to a plastic bottle. Both suspended sediment and filtered water samples were stored frozen (-15 °C) pending analysis.

Azadirachtin was extracted from aqueous samples by passing 200 mL aliquots through pre-conditioned C-18 solid phase extraction cartridges (Waters Sep-Pak Plus, Part No WAT020515, Waters Corp., Milford, Ma) under vacuum and eluting with 2 mL of acetonitrile. Eluates were collected in a 10 ml graduated centrifuge tube and evaporated under nitrogen (Meyer N-EVAP Analytical Evaporator, Model No 112, Organomation Associates Inc., South Berlin, MA,) to approximately 0.5 ml before being brought to an exact final volume (0.5 to 2 mL depending upon sample) with acetonitrile. Suspended sediment samples were subjected to accelerated solvent extraction (Dionex ASE 200, Dionex Ltd.) with acetone/water (70:30) v/v under high pressure (1000 psi) and moderate temperature (40 °C), using 2 extraction cycles each with a 5 min static time.

The concentration of AZA-A in water and filtered suspended sediments was determined by reverse-phase HPLC following the general method of Sundaram and Curry (1993) and using a HP1090 liquid chromatograph (Hewlett Packard Canada Ltd., Mississauga, Ont.). Isolation of AZA-A was achieved on a 150 x 4.60 mm i.d. column (Phenomenex Prodigy 5um ODS (3) eluting with acetontrile:water (20:80) mobile phase under isocratic conditions at a flow rate of 1.0 ml/min. Quantitation was conducted by comparison to a verified external standard (Azadirachtin A (>95% purity), Sigma Chemical Co., St. Louis, MO) using photo-diode array detection with wavelength settings of 215 (excitation), 230 and 260 (reference) nm. Data acquisition, integration and analyses were performed using Hewlett Packard Chemstation software (version #A.08.01.783) running on a Kayak Pentium II computer.

Blank water samples (N=35) were fortified both in the field and in the laboratory at levels ranging from 0.02 – 1.0 mg/L to provide data for quality assurance (QA) and control (QC) analyses. Samples were handled and processed exactly as for water samples taken from the mesocosms, allowing assessment of potential loss during transport, storage, processing and analytical work up.

RESULTS AND DISCUSSION

Field QA samples showed mean recovery of 97.95% with coefficient of variation of 10.8% demonstrating good precision and maintenance of sample integrity during handling, transport and storage procedures used for field samples. Similarly, lab QC samples showed high recovery efficiency, good precision and reproducibility of analytical methods, with limits of detection (LOD) and quantitation (LOQ) for water samples of 0.002 mg/L and 0.02 mg/L respectively

and an overall mean of 98.1% recovery with coefficient of variation of 15.3%. All AZA-A concentrations reported in this paper were corrected for analytical recovery efficiency.

Analysis of aqueous samples taken from control mesocosms on the day of treatment and periodically thereafter in both first and second experiments showed no AZA-A concentration above detection thresholds (LOD = 0.002 mg/L) confirming that there was no cross-contamination between mesocosms. Initial concentrations of AZA-A observed in aqueous samples on the day of treatment (4 hr after application) closely approximated nominal calculated levels (Table 1).

Table 1. Nominal and observed mean (N=5) initial concentrations of AZA-A in treated mesocosms

Treatment	Nominal Total*	Nominal AZA-A*	Observed AZA-A*	STD	CV	% of Nominal
Control	0.000	0.000	0.000	0.000	0.0	NA
Overspray	0.035	0.026	0.028	0.002	7.7	109
Low	0.175	0.128	0.131	0.010	7.4	102
Medium (1^{st})	0.700	0.511	0.514	0.030	5.7	101
Medium (2^{nd})	0.700	0.511	0.690	0.050	7.1	135
High	1.750	1.280	1.553	0.098	3.9	121

*Concentrations in mg/L; where total refers to total of AZA-A and AZA-B and where nominal AZA-A concentration is calculated based on 73% of total in formulation (M. Dimock, Thermo Trilogy, personal communication). 1^{st} and 2^{nd} refer to first and second experiments respectively.

Relatively high concentrations observed for the second experiment may reflect inaccuracy in enclosure volume estimation or incomplete mixing in the water column within the 4 hr period between treatment and sampling. In the first experiment, aqueous AZA-A residues dissipated significantly ($p < 0.0001$) over time following linear kinetics in both overspray (r^2= 0.88) and medium (r^2=0. 0.90) treatment levels (Figure 1). Slope (± SE) values for linear regressions were b = -0.0005 ± 0.0001 and b = -0.0085 ± 0.0003 for overspray and medium treatments respectively. Corresponding times to 50% dissipation (DT_{50}) were 25.0 and 29.2 d. Very similar results (data not shown) were observed for the second experiment where dissipation in mesocosms treated at the medium application rate were again characterized by significant ($p < 0.0001$) linear declines (r^2 =0.93) with a slope (b = -0.0098 ± 0.0004) and an estimated DT_{50} of 24.7 d. Although, results suggest a slight concentration dependence in dissipation rate, no seasonal differences AZA-A dissipation were observed as a result of differential DOC, water colour, sunlight irradiation or temperature variables. In practical terms, the similarity of DT_{50} values suggest that under the conditions of these experiments of AZA-A dissipates at moderate rates following linear kinetics with little influence of seasonal variables. Relatively slow dissipation rates and

pseudo-first order linear kinetics as observed in these experiments are consistent with previous laboratory studies (Szeto and Wan 1996; Sundaram 1996) which demonstrate temperature-dependent, base-catalyzed hydrolysis of azadirachtin as the dominant mechanism of dissipation in natural waters. The latter author demonstrated that hydrolysis of formulated products is significantly slower than that of pure technical AZA-A.

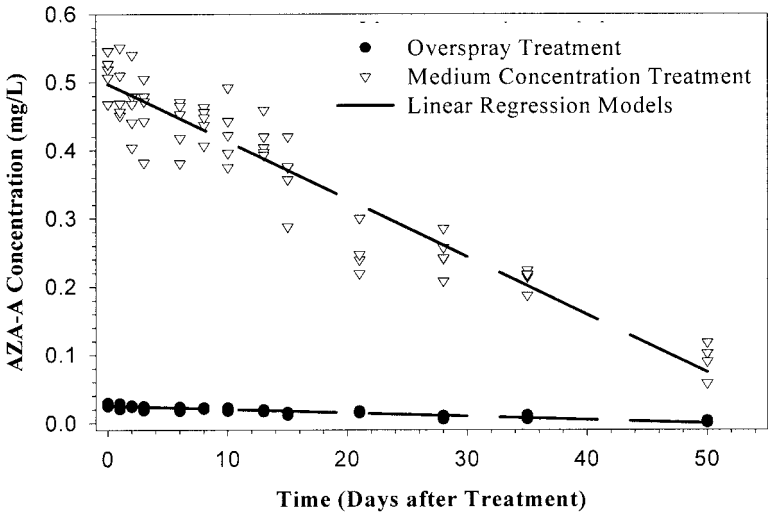

Figure 1. Dissipation of aqueous AZA-A residues in replicate (n=5) mesocosms treated with Neemix 4.5 at overspray and medium concentrations.

The DT_{50} estimates of 25 to 29 d observed in this study under conditions of natural sunlight irradiation, pH ~ 6.0, and temperature ~18 °C, are comparable to the estimate of 30.5 d reported by Sundaram (1996) under dark, pH =7, 20 °C laboratory conditions. Considering this relation, it appears that the combination of high DOC (4.5-6.0 mg/L), associated water colour, total suspended particulate matter (0.5-3.5 µg/L) and partial occlusion of the water surface by floating macrophytes in this natural lake system may have inhibited photolytic degradation by attenuating irradiation of the water column. The average DT50 values for AZA-A (~26 d) as observed in these experiments with Neemix 4.5 formulation differ dramatically from values of 36 to 48 hr reported for Neem-EC and Margosan-O formulations applied to small outdoor microcosms (Sundaram et al. 1997; Scott and Kaushik 2000). Sundaram et al. (1995) demonstrated that hydrolysis rates for AZA-A are highly dependent upon pH and that hydrolysis may be significantly retarded by surfactants in the formulation. The inhibition of aqueous hydrolysis at higher pH was confirmed by Szeto and Wan (1996), who

also showed that dissipation of azadirachtin was highly temperature dependent. Scott and Kaushik (2000) noted that azadirachtin formulations based on neem oil might remain on the surface layer of aqueous systems for several hours, thereby enhancing potential losses via volatilization and photolysis. Whether differences in aqueous persistence for the various studies noted above are the result of differential experimental systems, formulation effects, or environmental conditions (sunlight irradiation levels, pH, temperature, etc.) cannot be determined unequivocally. However, in contrast to the rapid dissipation observed under laboratory or more artificial test systems, our experiments suggest that AZA-A may be moderately persistent in natural forest lake, pond or wetland environments. Further research to confirm these findings and enhance understanding of key controlling variables is warranted.

In the first experiment, residues sorbed to suspended organic matter were occasionally quantifiable from 1 through 10 days after treatment (DAT) in mesocosms treated at the medium test level. No quantifiable sorbed residues were observed in mesocosms treated at the overspray (0.035 mg/L) concentration. AZA-A concentrations associated with suspended particulate matter were variable (Mean + STDEV (CV) = 16.26 ± 8.28 (54%) mg/L) and accounted for < 0.5% of total AZA-A concentrations in the water column in all cases. Similarly, in the second experiment, no significant differences (p = 0.984) were observed in AZA-A concentrations from filtered or unfiltered water within 4 hr after application. These results are consistent with the moderate water solubility, relatively low log Kow value (1.9) and the low and weak sorption for azadirachtin observed in both soils and aquatic bottom sediments (Sundaram 1996; Sundaram et al. 1997).

Results of this study indicate that following input to a typical Canadian forest lake, AZA-A remained primarily in dissolved phase with minimal sorption to suspended particulate matter. Consistent with base-catalyzed hydrolysis as the primary mechanism of degradation, dissipation occurred via linear kinetics with little to no concentration or seasonal dependence and with an average time to 50% dissipation approximating 26 d. As noted by Scott and Kaushik, (2000), partitioning properties of the compounds along with the functional feeding habits of the biotic species under investigation are important considerations in ecotoxicological investigations. The results of this experiment demonstrate that zooplankton, which were the focus of the overall study, were chronically exposed to diminishing levels of AZA-A and that any additional uptake and exposure associated with filter feeding activity on suspended particulate matter was negligible.

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