Bull. Environ. Contam. Toxicol. (1996) 56:432-439

Bulletin of Environmental Contamination and Toxicology

Evaluation of the Acute Toxicity to Juvenile Pacific Northwest Salmon of Azadirachtin, Neem Extract, and Neem-Based Products

M. T. Wan,[1] R. G. Watts,[1] M. B. Isman,[2] R. Strub[1]

[1]Environmental Protection Branch, Environment Canada, Pacific & Yukon Region, 224 West Esplanade, North Vancouver, British Columbia, Canada V7M 3H7
[2]Department of Plant Science, University of British Columbia, Vancouver, British Columbia, Canada V6T 1ZA

Received: 17 January 1995/Accepted: 26 August 1995

Azadirachtin (AZA; cas #:11141-17-6) is a natural bioactive agent derived from the seeds of the neem tree *Azadirachta indica* A. Juss (Saxena 1990). Neem seeds contain up to 40% by weight of oil, which can be expressed by cold pressing. Solvent extraction of neem seed cake (the residue remaining after oil expulsion) produces neem extract (NEX), which contains a mixture of more than 30 limonoid compounds in varying quantities but generally < 0.05%, except for AZA (Jones et al. 1988). Depending on the genome of the neem tree from which the seeds are collected, the AZA content of seed oil can range from < 0.05% to > 4% (Isman et al. 1990; NRC 1992). Proprietary partition and recrystalization processes can enrich the AZA content to 30% or greater. Neem-based products are commercial preparations of formulated NEX for sale as insecticides.

Unlike synthetic insecticides, which are mostly contact neurotoxins, AZA affects the endocrine system (feeding, development, reproduction and the general metabolism) of insects (Mordue and Blackwell 1993). In 1993, neem-based products were registered in U.S.A. for insect pest control of food and non-food crops, despite data gaps on fish toxicity. Although not registered in Canada as of yet, experimental field trials are presently being conducted in British Columbia to evaluate the efficacy of neem-based products for the control of insect pests in agriculture and forestry. As well, there is an interest to develop appropriate neem-based formulations for mosquito control. The objective of this study was to evaluate the acute toxicity to juvenile Pacific Northwest salmon of AZA, NEX, and neem-based products.

MATERIALS AND METHODS

The common name, abbreviation, and concentration of test

Correspondence to: M. T. Wan

Table 1. List of test materials

Common name	Abbrev.	Conc. of active ingredient
Azadirachtin	AZA	49% AZA, 51% ONC[1]
Azatin blank[2]	AZT-A	naphthalene/butanol-based solvent
Azatin™ EC[3]	AZT	3% AZA, ~ 27% ONC, 70% AZT-A
Margosan blank[2]	MAR-B	polyoxyethylene solvent + non-ionic surfactant
Margosan O[3]	MAR	0.3% AZA, ~ 12% ONC, 87.7% MAR-B
Neem extract[3]	NEX	23% AZA, 77% ONC
Pherotech blank[4]	PHE-C	alcohol-based solvent + emulsifier
Pherotech[4]	PHE	4.6% AZA,15% ONC,79.4% PHE-C

[1] - ONC = other neem constituents (including salanin, eacetyl salanin, salannol acetate, nimbin, deacetyl nimbin, nimbandiol, 6-acetylnimbandiol); [2] - Quarles 1994; [3] - based on active ingredients stated in product label; [4] - *per com* Phero Tech Inc., Delta, British Columbia, Canada

materials are summarized in Table 1. All test materials were obtained commercially, with the exception of AZA, PHE and its emulsifier, (PHE-C), which were supplied by the Department of Plant Science, University of British Columbia, Vancouver, British Columbia (B.C.).

A series of 96-h static acute toxicity tests was conducted from January to May 1994 in accordance with the protocols of Environment Canada (1990) and the procedure outlined in Wan et al. (1988). Juvenile Pacific Northwest salmon (Table 4) were obtained in fresh water from B.C. hatcheries and used as test fish. The average lengths of fish, weights, loading densities and test volumes were recorded. Ten fish were placed in each vessel and dosed once. Duplicate tests were conducted for each test concentration. Cumulative fish mortality was recorded daily and the LC50 values were calculated each day to observe the toxic effects of each material as the test progressed, using the "Lethal" computer program developed by Stephan (1983).

A volume of 20 L dechlorinated Vancouver City tap water was used in each test vessel. In the test series of AZA and NEX, 25 mL acetone was used as the carrier, and an equal amount of acetone was added to the corresponding control test vessels. The dilution water was analyzed three times to check its quality, and once for chlorinated pesticide residues. A 250-mL water sample was cornposited from ten 25-mL aliquots taken at random from the 1 mg/L AZA test concentration of the coho test vessel to ascertain the AZA content. Sampling started after the addition of AZA and the 30-min preaeration process and continued daily. Water samples were stored at 4°C until

the completion of the 96-h test, and then extracted and analyzed for AZA residues.

The screening of water samples for extractable metals, nutrients, and chlorinated pesticides was conducted at Environment Canada Laboratory, West Vancouver, B.C., using procedures outlined in Environment Canada (1989). Residues of AZA in water were determined in the same laboratory, using a modified method of Sundaram and Curry (1993) developed in-house. The sample was first extracted with methylene chloride, filtered, and then concentrated. Acetonitrile was added to the concentrated extract, which was then analyzed for AZA residues, using High Performance Liquid Chromatography (HPLC). The detection limit was 0.02 mg/L. The 24, 48, 72 and 96-h LC50 values for the test materials were not adjusted to the measured AZA concentration.

RESULTS AND DISCUSSION

The quality of Vancouver City dilution water was relatively consistent throughout the study period from January to May, 1994 (Table 2). No detectable residues (detection limit, 1 µg/L) were found in the water for the following chlorinated pesticides: DDT analogues, benzene hexachloride, cyclodiene and phenoxy compounds, PCB, PCP, and picloram.

The measured concentration of AZA in the coho test vessel before and after fish introduction was less than the nominal concentration (Table 3). This finding suggests that: (1) there was a 35% AZA loss probably via volatilization (Wan et al. 1988), (2) AZA did not adsorb onto glass surfaces of the test vessel, as no residues were found in the acetone rinse, and (3) AZA remained relatively stable during the 96-h period in slightly acidic water (pH = 6.7) at 12°C ± 2°C. It was assumed that AZA of the other test materials would behave similarly in water.

No signs of stress, e.g., coughing, physical disorientation nor state of morbidity, of coho test fish were observed at the highest test concentration of 4 mg/L AZA (49% purity). This indicated that the 96-h LC50 for the fish of AZA was > 4 mg/L (Table 4). Further LC50 tests using pure AZA are not being planned due to the high cost of obtaining the material. To date, synthetic AZA is not available. The results also show that the LC50 values for salmon changed during the test period for various test materials. The LC50 change at the end of the 96-h exposure varied from 8% to > 41 % for different test materials (Table 4), indicating the differences in degree of cumulative toxicity of the different test materials.

Table 2. Quality[1] of Vancouver City dilution water

Parameter analysed	Concentration
Alkalinity ($CaCO_3$)	2.7 ± 0.3
Chemical elements/ions	
C (total)	4.5 ± 1.1
Ca	1.4 ± 0.1
K	0.1
Mg	0.2
Na	0.8 ± 0.1
Si	1.5 ± 0.1
Cl	1.7 ± 0.8
SO_4	3.3 ± 0.3
Conductivity (umhos/cm)	19 ± 0.7
ardness (total as $CaCO_3$)	5.5 ± 0.3
pH (rel. Units)	6.7 ± 0.1

[1] - parameter measured in mg/L (mean ± S.E.; n = 3); detection limits, < 0.001 - 0.01 mg/L

Table 3. Stability of azadirachtin in water of test vessel

Sampling time (h)	Sample type	AZA concentration (mg/L)	
		nominal	measured
0	water	0.49	0.31
24	water	0.49	0.35
48	water	0.49	0.31
72	water[1]	0.49	N/A[1]
96	water	0.49	0.30
96	acetone rinse[2]	N/D[3]	N/D[3]

[1] - N/A = not available, water sample contaminated; * - 200 mL acetone rinse of empty test vessel; [3] - N/D = not detected, detection limit = 0.02 mg/L

The order of decreasing acute toxicity to juvenile salmon of test materials is as follows: AZT > NEX > AZT-A > MAR > PHE > PHE-C > MAR-B (Table 5). AZT was significantly more toxic to young salmon than MAR (8 times), PHE (18 times) and PHE-C (25 times). Likewise, NEX was acutely more toxic than MAR (5 times), PHE (10 times) and PHE-C (14 times). It was however, not significantly more toxic to fish than AZT despite having 7.7 times more AZA. MAR was twice and three times more toxic to salmon than PHE and PHE-C, respectively. Although PHE contained higher AZA content, i.e., 1.5 and 15 times more than AZT and MAR, respectively, it was the least toxic product to young salmon of the three neem-based materials. The cause of this difference in fish toxicity of these products was most likely due to the carrier solvent used in the respective

Table 4. Acute toxicities to juvenile salmon of azadirachtin, neem extract, and neem-based products

Test chemical	Test Fish[1] Species	LC50[2] (mg/L) 24-h	48-h	72-h	96-h	Change of LC50 (%) 24 to 96-h
AZA	Coho	> 4	> 4	> 4	> 4	0
AZT-A	Rainbow	14	14	14	14	0
AZT	Chinook	5	5	5	4	20
	Coho	11	5	5	5	55
	Rainbow	7	5	5	4	43
			Mean ± S.E. =		4 ± 0.4	39
MAR-B	Rainbow	> 520	> 520	> 520	> 520	0
MAR	Chinook	> 60	37	37	31	> 48
	Coho	> 50	48	39	38	> 24
	Rainbow	61	37	29	29	52
			Mean ± S.E. =		33 ± 3	> 41
NEX	Chinook	6	4	4	4	33
	Coho	23	16	14	13	43
	Rainbow	4	4	3	3	25
			Mean ± S.E. =		7 ± 3	34
PHE-C	Chinook	57	57	57	55	4
	Coho	> 130	123	120	117	> 10
	Rainbow	137	129	129	127	7
			Mean ± S.E. =		100 ± 39	> 8
PHE	Chinook	95	80	77	74	22
	Coho	> 95	91	89	81	15
	Rainbow	86	75	68	61	29
			Mean ± S.E. =		72 ± 6	22

[1] - chinook (*Onchorhynchus tshawytscha*), coho (*O. kisutch*), Rainbow trout (*O. mykiss*); age = 2.5 + 0.3 mo., length = 4.7 ± 0.3 cm, 50 fish; weight 0.5 ± 0.1 g, 50 fish; loading density = 0.1 - 0.3 g/L; [2] - nearest whole number

formulations. AZT-A appeared to increase the AZT toxicity to salmon, whereas PHE-C did not significantly (Table 5) influence PHE. In fact, PHE-C was about 1.4 times less toxic to the fish than PHE. The trend of decreasing toxicity to salmon of the three emulsifiers used in these products was as follows: AZT-A > PHE-C > MAR-B. The influence of MAR-B on MAR's effect on salmon toxicity, however, appeared to be much greater than AZT-A and PHE-C.

The effect of varying quantities of ONC (Table 1) found in

Table 5. Order of decreasng toxicity to salmon of test materials

Test Materials	Toxicity 96-h LC50 (Mean+S.E.)	No. of species	Student "t" test	"t" value	Level of signif. (P value)
1. AZT	4 ± 0.4	3	1 vs. 2	0.99	n.s.
			1 vs. 4	9.58	<0.01
			1 vs. 5	11.31	<0.01
			1 vs. 6	2.46	<0.10
2. NEX	7 ± 3	3	2 vs. 4	6.13	<0.01
			2 vs. 5	9.69	<0.01
			2 vs. 6	2.38	<0.10
3. AZT-A	14	1	-	-	-
4. MAR	33 ± 3	3	4 vs. 5	5.81	<0.01
			4 vs. 6	1.71	<0.20
5. PHE	72 ± 6	3	5 vs. 6	0.71	n.s.
6. PHE-C	100 ± 39	3			
7. MAR-B	>520	1	-	-	-

n.s. = not significant

Table 6. Comparison of acute toxicity to salmon of azadirachtin, neem extract and neem-based products with some common insecticides

Pesticide[1]	Acute toxicity[2] 96-h LC50 (mg/l)	
	Coho	Rainbow
AZA (49%)	> 4	N/A
Azinphosmethyl (94%)	0.006	0.004
AZT (3% AZA)	5	4
Carbofuran (99%)	0.5	0.4
Diazinon (89%)	N/A	0.09
Endosulfan (96%)	N/A	0.0001
Malathion (95%)	0.2	0.2
MAR (0.3% AZA)	38	29
NEX (23% AZA)	13	3
PHE (4.6% AZA)	81	61
Pyrethrum (20%)	0.03	0.04
Rotenone (44%)	N/A	0.03

[1] - % active ingredient; [2] - Johnston and Finley (1980), Pimentel (1971); N/A - not available

each test material on salmon toxicity is unclear. For example, NEX (23% AZA, 77% ONC) was highly toxic to salmon (96-h LC50 = 7 mg/L), whereas AZA (49% purity, 51% ONC)) did not appear to be nearly as toxic (96-h LC50 > 4 mg/L). Test records showed that there was a 30% fish mortality observed for NEX at a test concentration of 3 mg/L, while young salmon

subjected to a 4 mg/L AZA test concentration displayed no signs of stress nor state of moribund.

When compared to some of the common synthetic insecticides such as carbamates (carbofuran), organochlorine (endosulfan), and organo-phosphates (azinphosmethyl, diazinon, malathion), AZA and neem-based products appeared to be far less toxic to young salmon (Table 6). They also have a greater margin of safety to juvenile salmon than some of the natural neurotoxic botanical insecticides such as pyrethrum and rotenone.

Under field conditions, the concentration of neem-based insecticides in a stream unintentionally oversprayed during an aerial or ground-based operation would unlikely exceed 0.05 mg/L in 15 cm water, even at the highest rate recommended (0.06 kg active ingredient/ ha), except perhaps during an accidental spill. The potential of neem-based products causing fish kills is therefore small when they are used under product labelled conditions. However, the possibility of these materials having sublethal effects on young salmon is presently not known.

In summary, the 96-h LC50 value of AZA (49 % purity) for juvenile salmon is greater than 4 mg/L. NEX is the most toxic neem material to young salmon. The toxicity of neem-based products (AZT, MAR, and PHE) to the fish, however, depends on the solvents and emulsifiers used in formulating the materials, with 96-h LC50 values ranging from 4 mg/L to 72 mg/L. The role of ONC in fish toxicity is unclear. Neem-based products have a greater margin of safety to young salmon when compared to some common synthetic and botanical insecticides.

Acknowledgments. We thank (1) B. Kelso and L. Churchland (Environmental Protection Branch, Environmenat Canada) for their comments, encouragement and support; (2) S. Samis (Fisheries & Oceans Canada), S. Szeto (Agriculture Canada) for their comments;(3) W.R. Grace & Co, Connecticutt, and Agridyne Tech. Inc., Utah, for supplying test samples; and (4) M. Fennell, D. Moul, S. Yee, and G. van Aggelen for technical support.

REFERENCES

Environment Canada (1990) Biological test method: acute lethality test using rainbow trout. Environmental Protection, Ottawa, Ontario. EPS 1/RM/9. Beauregard Printers Ltd., Ottawa, Canada, 51 pp

Environment Canada (1989) Metals and water; organo-chlorine pesticides screening. In: Conservation and Protection

Laboratory Manual, Vancouver, British Columbia, Pacific & Yukon Region, p 1.1-1.8, 7.1-1.14
Isman MB, Koul O, Luczynski A, Kaminski J (1990) Insecticidal and antifeedant bioactivities of neem oils and their relationship to azadirachtin content. J Agric Food Chem 38:1406-1411
Jones PS, Ley SV, Morgan ED, Santafianos D (1988) The chemistry of the neem tree. p 19-46. In: M Jacobson (ed) Focus on photochemicals pesticides. I: the neem tree. CRC Press, Inc, Boca Raton, Florida
Johnston WW, Finley MT (1980) Handbook of acute toxicity of chemicals to fish and aquatic invertebrates. US Department of the Interior Fish and Wildlife Service Resource Publication 137, Washington, DC
Quarles W (1994) Neem tree pesticides protect ornamantal plants. The IPM Practioner. XVI(lO) :l-13
Mordue AJ, Blackwell A (1993) Azadirachtin: an update. J Insect Physiol 39:903-924
National Research Council (1992) Neem: a tree for solving global problems. National Academy Press, Washington, DC, 141 pp
Pimentel D (1971) Ecological effects of pesticides on non-target species. Executive Office of the President Office of Science and Technology, Washington, DC
Saxena RC (1990) Insecticides from Neem. In: Arnason JT, Philogene BJR, Morand P (eds.) Insecticide of plant origin. ACS Symp Ser 387 American Chemical Society, Washington, DC, pp 110-135
Stephan CE (1983) Lethal program for computer analyses of LC50. Environmental Research Laboratory, U.S. Environmental Protection Agency, Duluth, Minnesota
Sundaram KMS, Curry J (1993) High performance liquid chromatographic determination of azadirachtin in conifer and deciduous foliage, forest soils, leaf litter and stream water. J Liquid Chromat 16:3275-3290
Wan MT, Watts RG Moul DJ (1988) Evaluation of the acute toxicity to juvenile Pacific salmonids of hexazinone and its formulated products: Pronone l0G, Velpar® L, and their carriers. Bull Environ Contam Toxicol 41:609-616