The Acute Toxicity of Four Herbicides to 0-4 Hour Nauplii of *Cyclops Vernalis* Fisher (Copepoda, Cyclopoida)[1]

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A number of aquatic crustaceans have been used as assay organisms for testing the effects of herbicides in aquatic systems. The cladocerans Daphnia magna and D. pulex and a number of amphipod and isopod species have been predominant in these roles. Among the common aquatic crustaceans, the copepods have been the most neglected in bioassay, with only adult animals being used in most of the copepod studies conducted.

ADAMS (1927) found the chemicals used in water treatment were lethal to Cyclops sp. and Daphnia sp. at levels below 5.0 ppm. KONAR (1970) showed that the insecticide heptachlor was toxic to Cyclops sp., Diaptomus sp. and nauplii at 0.1 ppm. RIO (1971) studied the survival and reproduction of Cyclops vernalis Fisher exposed to Abate, an organophosphorus mosquito larvicide, and three selected environmental factors. Survival and reproduction were found to be altered by the Abate and its interactions with the environmental factors. NAQVI and FERGUSON (1971) used six species of cyclopoid copepods, including C. vernalis, to demonstrate increased tolerance of copepods to insecticides caused by prior exposure. It was found that the animals which had had prior exposure were more resistant than those which had not. No indication was given as to variation in resistance between the species. BAUDOUIN and SCOPPA (1974) found that adult Cyclops abyssorum prealpinus (Einsle) and Eudiaptomus padanus padanus (Burkhard) were more tolerant to various metal salts than adult Daphnia hyalina (Leydig). The calanoid E. padanus padanus was shown to be more sensitive to the metals than the cyclopoid C. abyssorum prealpinus.

This study was undertaken to determine the acute toxicity of four herbicides to a naupliar copepod. A comparison of the toxicities of reagent and commercial grades of herbicides was also made.

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Materials and Methods

Test animal

The test organisms were 0-4 hour nauplii of the copepod Cyclops vernalis Fisher. Cyclops vernalis was selected because it is abundant and found throughout North America; is easily reared in the laboratory; has a short generation time (COKER 1934); and is found in areas where nuisance plant control has been attempted, notably in the reservoirs of the Tennessee Valley Authority (BUNTING 1973). The use of naupliar copepods was desirable because early developmental stages have been shown to be more sensitive to toxic materials than the adults (MARTIN 1973).

Cyclops vernalis were mass cultured in the laboratory in the three-salt standard test medium (STM) described by FREAR and BOYD (1967). Water characteristics were as follows: pH 6.7, calcium hardness ($CaCO_3$) 70 ppm, total dissolved solids 330 ppm, and alkalinity ($CaCO_3$) 31.6 ppm. The C. vernalis were maintained on a mixed algal culture consisting primarily of Chlorella spp. and Scenedesmus spp. with nauplii of the brine shrimp, Artemia sp., being used as a supplemental food.

The herbicides

The herbicides used in the tests were amitrole, amitrole-T, the free acid of 2,4-D, and the alkanolamine salt of 2,4-D. The amitrole and amitrole-T were supplied by Amchem Products Incorporated of Ambler, Pennsylvania. The amitrole was reagent grade while the amitrole-T was commercial formulation containing 20 percent active ingredient. Amitrole-T has had ammonium thiocyanate added to the amitrole to act as an activator.

The formulations of 2,4-D were supplied by Dow Chemical Company of Midland, Michigan. The alkanolamine salt was Dow Chemical's commercial grade Formula 40 herbicide with an acid equivalent of 38.6 percent. The free acid was a reagent grade herbicide.

Test method

Static bioassays were conducted in Cooke serological microtiter plates so that microscopic examination of individual nauplii could be made. Fifty concavities, each containing 25 ml, were used on each plate. Fifty 0-4 hour nauplii were randomly selected with no more than five nauplii from one female being used at any one concentration. The limited number of nauplii from each female reduced the possibility of genetic bias. Tests were conducted at 20°C in an incubator with a 16-8 hr.

photoperiod. Each test series had a duration of 96 hours, with mortality observed at 12-hr. intervals. Immobilization was the criterion of response and was determined by the inability of the nauplii to respond to tactile stimulation. The nauplii were not fed during the tests.

Data evaluation

Immobilization data were analyzed by computer using a program of probit analysis written by Richard J. Daum and Wallace Killcreas of the Entomology Research Division of Mississippi State University. The program uses a maximum likelihood procedure to calculate a weighted linear regression of percent mortality in probit values on the log of the dose. Correction for natural mortality in the controls was made using Abbott's formula. Data input for the determination of median lethal times (LT50) was composed of time in hours as dose, the number of animals exposed, and the number of animals responding. To calculate the 48- and 96-hour median lethal dose (LD50), the concentration of herbicide was used as the dose input.

Data output consisted of LD values from 10 to 99 percent mortality along with their associated confidence limits, and the necessary statistics for computing a regression line for each data set.

The slopes of the regression lines for the time-mortality data were compared to determine if changes in mode of toxic action were detectable. This method was similar to that described by SPRAGUE (1969) where the slopes of lines plotted on log-probit paper were compared to determine changes in mode of action.

Results and Discussion

A comparison of the toxicities of the four herbicides is shown in Table I. It may be seen that marked differences in the toxicities of different formulations of the herbicides were obtained. Amitrole-T was found to be the most toxic of the herbicides tested with 48- and 96-hr. LD50's of 2.32 and 2.04 ppm respectively. The 2,4-D (salt) proved to be the least toxic of the four with LD50's of 662.1 and 142.0 ppm.

A comparison of the toxicity of amitrole and amitrole-T indicated that the amitrole-T was roughly 25 times more toxic at the end of 48 hours and 10 times as toxic at 96 hours. In terms of amount of active ingredient, the relative toxicity of the amitrole-T was 127 times greater at 48 hours and 48 times greater at 96 hours.

CROSBY and TUCKER (1966) found that amitrole was more toxic to Daphnia magna than was amitrole-T. It was shown that the 26-hr. LD50 for amitrole was 23.00 ppm while amitrole-T yielded a LD50 of

TABLE I

48- AND 96-HOUR LD50 VALUES FOR 0-4 HOUR NAUPLII OF CYCLOPS VERNALIS AT 20°C

	LD50 (ppm)	
	48 hour	96 hour
Amitrole	58.5 (39.76-124.0)	22.1 (18.54-27.71)
Amitrole-T[a]	2.32 (1.81-3.86)	2.04 (1.89-2.41)
	0.49[b] (0.38-8.81)	0.43[b] (0.40-0.51)
2,4-D (Free Acid)[a]	37.42 (308080.8-21.7)	8.72 (5.32-11.57)
2,4-D (Alkanolamine Salt)	662.11 (16118.3-329.8)	142.0 (120.34-167.3)
	225.57[c] (6221.66-127.3)	54.8[c] (46.45-64.6)

a. Tested at 10 percent level.
b. Expressed in terms of active ingredient.
c. Expressed in terms of acid equivalent.
() Lower and upper limits.

40.00 ppm in 26 hours. FINDLEY (1969) obtained a similar LD50 for aminotriazole, a formulation of amitrole containing 50 percent active ingredient, and D. magna. SANDERS (1970) reported a 48-hr. LD50 for amitrole-T using D. magna of 30.00 ppm. Cyclops vernalis, while somewhat more tolerant to amitrole than D. magna, exhibits a much greater sensitivity to amitrole-T. This may be due to a specific lack of tolerance to the presence of ammonium thiocyanate in amitrole-T. It is possible that plasticizers used to make the container in which the amitrole-T was supplied may have contaminated the herbicide resulting in the higher toxicity. SANDERS, et al. (1973) have shown some common plasticizers to have 96-hr. median lethal tolerance limits of 2.1 mg/l to some aquatic crustaceans. Interactions between the amitrole, ammonium thiocyanate, and the plasticizers may have caused the high toxicity of amitrole-T to C. vernalis.

The free acid of 2,4-D was shown to be more toxic to C. vernalis than the alkanolamine salt. A comparison of 48-hr. LD50's indicated that the 2,4-D (acid) was nearly 18 times more toxic than the 2,4-D (salt). At the end of 96 hours the 2,4-D (acid)

toxicity exceeded that of the salt by some 16 times. Toxicity expressed in terms of acid equivalents resulted in the relative toxicity of the acid being reduced to about seven times that of the salt at both time intervals.

The nauplii of C. vernalis were found to be one of the most sensitive aquatic crustaceans when exposed to the 2,4-D (acid). CROSBY and TUCKER (1966), and SANDERS (1970) each showed Daphnia magna to be resistant to concentrations of the free acid exceeding 100.0 ppm. SANDERS (1970) found the amphipod Gammarus fasciatus the most sensitive crustacean tested with a 48-hr. LD50 of 3.2 ppm.

MEYER (1966) reported test specimens of the bluegill Lepomis macrochirus Rafinesque were all killed by 40 ppm of the alkanolamine salt of 2,4-D. This indicates that C. vernalis is much more resistant to 2,4-D (salt) than L. macrochirus. No comparitive data on the toxicity of 2,4-D (salt) to other crustaceans was available.

Table II is composed of the LT50's and the regression equations for the time-mortality data at each concentration. An examination of the slopes of the equations shows that a definite alteration in slope between 15.0 and 20.0 ppm of amitrole. This indicates that there possibly were two modes of toxic action for amitrole. Likewise, amitrole-T displayed a single distinct change of slope.

The free acid of 2,4-D exhibited two marked slope changes between 5.0 and 10.0 ppm and between 10.0 and 20.0 ppm. This indicates three possible modes of action. The alkanolamine salt displayed two modes of action denoted by the break in slope between 200.0 and 400.0 ppm. It was also noted that mortality exceeding one percent among those animals exposed to 2,4-D (salt) at all concentrations did not occur until the end of 48 hours.

The animals exposed to amitrole and amitrole-T did not exhibit any outstanding changes in behavior prior to death or in sublethal concentrations. The bodies of the nauplii killed by both herbicides developed a white opacity.

Animals exposed to the 2,4-D (salt) developed a swimming impairment prior to death. Some 12 hours prior to death the nauplii would cease to swim and commenced to crawl about the bottom of the concavity. The free acid did not produce such impairment of swimming. The free acid and the salt each caused the appearance of a protuberance on the posterior of each nauplius. This may have been a molted exoskeleton which was incompletely cast off, a herniated gut, or gut contents expelled at death. Unlike the forms of amitrole the alkanolamine salt caused a clearing of the bodies at death.

Conclusions

1) When compared with published data Cyclops vernalis Fisher was found to be among the most sensitive crustaceans when exposed to amitrole-T and 2,4-D (free acid).

TABLE II

MEDIAN LETHAL TIMES FOR 0-4 HOUR NAUPLII OF CYCLOPS VERNALIS AT 20°C

Herbicide	Concentration (ppm)	Median Lethal Time (hours)		Regression Equation
Amitrole	15.0	105.12	(92.46-138.14)	y=-4.32+4.610(x)
	20.0	143.65	(104.68-303.30)	y=0.876+1.900(x)
	25.0	92.54	(78.31-122.40)	y=0.203+2.440(x)
	40.0	50.18	(41.45-60.92)	y=1.932+1.804(x)
Amitrole-T	2.0	139.92	(87.99-589.90)	y=2.850+1.002(x)
	5.0	12.84	(10.13-15.13)	y=0.372+4.170(x)
	10.0	11.72	(8.52-14.20)	y=0.433+4.270(x)
2,4-D (Free Acid)	5.0	110.12	(95.57-344.41)	y=-10.54+7.61(x)
	10.0	81.34	(74.90-90.48)	y=-3.130+4.26(x)
	20.0	95.18	(66.57-467.80)	y=0.791+2.120(x)
	40.0	36.11	(31.49-41.40)	y=1.469+2.260(x)
2,4-D (Salt)	100.0	102.77	(92.36-124.80)	y=-4.43+4.450(x)
	200.0	83.51	(78.01-89.80)	y=-3.58+4.470(x)
	400.0	64.73	(60.00-69.19)	y=-8.05+7.200(x)
	800.0	51.22	(43.10-57.49)	y=-9.89+8.703(x)

( ) Lower and upper 95 percent confidence limits

2) The toxicity of amitrole to C. vernalis was somewhat less than published data for Daphnia magna. The 2,4-D (salt) was shown to be less toxic to C. vernalis than reported toxicity for Lepomis macrochirus.

3) The toxicity of the commercial formulations was not dependent on the active ingredient content. Amitrole-T with 20 percent active ingredient proved to be more toxic than reagent grade amitrole. The alkanolamine salt of 2,4-D was less toxic than 2,4-D (free acid) to an extent much greater than could be expected from the examination of the acid equivalent of 2,4-D (salt).

4) Each of the herbicides examined apparently exhibited more than one mode of toxic action. This was demonstrated by the time-mortality data.

5) The method used here would be useful in the determination of effects other than mortality such as instar lengths, rates of molting and susceptability of different naupliar stages to stress factors.

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