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Reproducibility of a Life-Cycle Toxicity Test with *Daphnia magna*

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The Toxic Substances Control Act (TSCA 1976) and the Resource Conservation and Recovery Act (RCRA 1976) require that effects on aquatic biota from chronic exposure to potentially toxic materials be determined. Standardized chronic life-cycle toxicity testing procedures with aquatic species are being developed (ASTM 1978). These tests will facilitate comparisons of toxicity data among different test species and among different toxic chemicals.

In chronic toxicity testing, an end point that has been used widely to assess the relative hazard of a toxic chemical is the no-observed-effects concentration (NOEC) (MAKI 1979) or the maximum-acceptable-toxicant concentration (MATC) (MOUNT & STEPHAN 1967). Both are defined as the highest concentration of a chemical or other material that causes no statistically significant observed effects on the test organisms compared with the control (MAKI 1979). Because the statistical determination of both of these end points is based upon the nonrejection of a hypothesis (i.e., no effect), and the scientific method generally involves the rejection of a hypothesis, SKALSKI 1979 suggested that an end point scientifically better than the NOEC would be one whose determination involved the rejection of a hypothesis. Such an end point would be the lowest concentration that produces significant toxic effects compared to controls. SKALSKI termed this the "lowest rejected concentration tested (LRCT)."

The cladoceran, Daphnia magna, and the fathead minnow, Pimephales promelas, have been used widely in life-cycle toxicity testing, because they are easy to work with and they are sensitive to many toxic chemicals (ANDERSON 1944, MCKEE & WOLF 1963, KENAGA 1978, MAKI 1979). NOECs estimated for chemicals on both species from life-cycle toxicity tests are closely correlated (MAKI 1979). Because life-cycle toxicity tests with D. magna require fewer than 30 d compared with nine months for tests with fathead minnows and because the former tests cost less and need fewer materials, they are usually the tests of choice.

Two widely used life-cycle toxicity tests with D. magna are the static-renewal method (ASTM 1978) and the flow-through method (MAKI 1977), but there is little information on

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reproducibility of chronic toxicity end points using these tests or on the relative sensitivity and variability of the toxicity criteria used to determine the end points. Our objectives were to determine if LRCTs obtained for six different toxicity criteria in static-renewal tests with the chemical acridine and D. magna were reproducible over time and to determine the relative sensitivity and vari- ability of the toxicity criteria. We chose the azaarene, acridine, because of our experience with it in toxicity tests with components of coal conversion products and wastes, and because of its relative persistence in aqueous solution.

MATERIALS AND METHODS

Our methods were generally those recommended by the ASTM (1978) for chronic toxicity tests with D. magna. First instar animals less than 24 h old were exposed to acridine concentrations of 0.2, 0.4, 0.8, 1.6, and 3.2 mg/L. The test solutions were prepared by dissolving a small quantity of acridine in a small volume of methanol and then adding this solution to well water to give the highest test concentration. Lower concentrations were obtained by serial dilution. The pH of the dilution water was 7.8; its alkalinity was 120 mg/L; its hardness was 140 mg/L. In the test solutions, methanol concentrations never exceeded 1 mL/L. Animals were exposed singly for 28 d to 50 mL of test solution in 100-mL covered glass beakers. Water temperature was maintained at 20 ± 0.5 C. Lighting was controlled with alternate 12-h light-dark periods. The animals were transferred to fresh test solutions thrice weekly, and at those times 1 mg of filtered homogenized trout chow was added. At the time of transfer, the number of survivors, broods produced, and young produced were recorded. Acridine concentrations were monitored throughout each test with a spectrophotometer. Twenty animals were used for each test concentration. Control animals were exposed to well water and well water with a methanol concentration of 1 mL/L. The tests were repeated four times at 3-mo intervals under conditions as nearly identical as possible.

Toxicity criteria used were survival, two indices of maturation, and three indices of reproduction. Survival was measured over a 28-d period of exposure. Maturation was defined as: (1) the occurrence of the primiparous instar which is defined as the instar at which young are first present in the brood chamber of the female, and (2) the age when young were first released. Reproductive effects were judged by: (1) the number of broods produced per female, (2) the number of young per brood, and (3) the total number of young produced per female during the test period. LRCTs were determined within each replicate test for each of the six toxicity criteria. Analysis of variance and Duncan's Multiple Range Test, as implemented in the Statistical Analysis System (SAS INSTITUTE 1979), were employed for each toxicity criterion to identify LRCTs, using a

Type I error level (SOKAL & ROHLF 1969) of 0.05. The variability of the toxicity critieria in each of the four tests was evaluated using analysis of variance and Duncan's Multiple Range Test. In addition, means ($\bar{Y}$), standard deviations (S), and coefficients of variation ($S \times 100/\bar{Y}$) were calculated. The null hypothesis tested was that criterion means would not vary significantly when the tests were repeated.

RESULTS AND DISCUSSION

Acridine Concentrations. Acridine concentrations were not found to change measurably between renewals in any of the four tests.

LRCTs Reproducibility. The estimated LRCTs ranged between 0.8 and 3.2 mg/L of acridine in the four tests (Table 1). The LRCTs estimated for the toxicity criteria of survival, age at onset of reproduction, and number of broods produced per female varied significantly among the four tests, and therefore, the LRCTs estimated from these toxicity criteria were not reproducible using our definition of reproducibility. However, the LRCTs estimated for occurrence of primiparous instar, number of young per brood, and number of young produced per female did not vary at all in the four tests; therefore, the LRCTs using these criteria were reproducible. The latter two criteria were the most sensitive for estimating the LRCT, since their LRCT (0.8 mg/L) was less than the value for occurrence of the primiparous instar (1.6 mg/L).

TABLE 1. Lowest rejected concentrations tested (LRCTs) from four tests on the chronic toxicity of acridine to *Daphnia magna* for each of six toxicity criteria

Toxicity criteria	LRCT (mg/L) Test number			
	1	2	3	4
Survival	1.6	3.2	0.8	1.6
Occurrence of primiparous instar	1.6	1.6	1.6	1.6
Age at onset of reproduction	1.6	1.6	0.8	0.8
Number of broods produced per female	1.6	0.8	0.8	1.6
Number of young per brood	0.8	0.8	0.8	0.8
Number of young produced per female	0.8	0.8	0.8	0.8

Toxicity Criteria Variability. The variability between the four replicate tests in the response of D. magna to 28-d exposure to the concentration of acridine used in this study is demonstrated in Tables 2-7. The mean values calculated for each toxicity criterion at each acridine concentration, including the two sets of controls, tended to vary significantly among the four tests. There were only a few exceptions to this tendency: (1) mean survival was not significantly different among the four tests at an acridine concentration of 0.2 mg/L and for the two sets of controls (Table 2); (2) the occurrence of the primiparous instar did not vary significantly in the methanol controls (Table 3); and (3) at 3.2 mg/L the means for all of the criteria involving reproduction of D. magna (all except survival) were zero, since none of the animals successfully reproduced at this concentration of acridine. Therefore, except as noted, the null hypothesis that criterion means were equal, was rejected. Inspection of the results in Tables 2-7 demonstrates that for any given concentration of acridine or for the controls the criterion mean for at least one test was generally substantially different in magnitude from the others in all cases where significant differences were found. In addition, CVs ranged from 5 to > 100% and were often > 20%. Therefore, it can be concluded that the rejection of the null hypothesis was apparently due to real differences between means and not just small differences associated with small variances that would probably not be biologically important. It is believed that the differences observed among the four tests in criterion means at nearly all concentrations of acridine reflect physiological differences in the groups of D. magna used in each of the four tests. Evidence for these physiological differences can be seen in the substantial and significantly large differences among the four tests in the numbers of young produced in control animals (Table 7) and in the apparent differences in sensitivity of D. magna to acridine in each of the four tests. For example, at 1.6 mg/L, the mean number of young produced per female was 4.2, 0.2, and 0.0, and 0.0, for tests 1, 2, 3, and 4, respectively. Therefore, the D. magna used in tests 3 and 4 were apparently more sensitive to acridine than the D. magna used in tests 1 and 2. Experimental error may have also attributed to some of the differences seen between the four tests. For example, CVs were lowest in Test 1 for all criteria except age at first brood release, and the CVs tended to increase going from test 1 to tests 3 and 4.

CVs also tended to increase with increasing acridine concentration for all toxicity criteria except age at first brood release. The increases in CVs were evident until concentrations of acridine were reached which completely inhibited reproduction, i.e., 1.6 mg/L for tests 3 and 4 and 3.2 mg/L for test 2.

The variability we observed in six toxicity criteria in four life-cycle toxicity tests with D. magna is similar to what other investigators have found. WINNER & FARRELL (1976)

repeated a test of the toxicity of copper to D. magna three times. Their test methods differed from ours principally in that their test was continued until all test organisms had died, while ours was stopped at 28 d. For their control animals, the range of differences in the total number of young produced among the tests was 42%, while the range of difference in mean brood size among the tests was 24%. Our range of differences among four tests for control animals was 55% for the mean number of young produced per female and 23% for mean brood size.

TABLE 2. Variability between four replicate tests in the effect of 28-d exposure to acridine on survival of Daphnia magna

Concentration (mg/L)	Survival (days)							
	Test 1		Test 2		Test 3		Test 4	
	$\bar{Y}$[a]	CV[b]	$\bar{Y}$	CV	$\bar{Y}$	CV	$\bar{Y}$	CV
0.0 (control)	28.0[c]	0.0	27.2[c]	6.8	28.0[c]	0.0	26.9[c]	25.6
0.0 (methanol control)	28.0[c]	0.0	26.8[c]	14.9	26.7[c]	16.2	26.8[c]	23.7
0.2	28.0[c]	0.0	27.9[c]	1.6	27.7[c]	3.4	28.3[c]	15.4
0.4	27.9[c]	1.6	27.9[c]	1.6	27.5[c]	6.8	25.2[d]	23.9
0.8	27.9[c]	1.6	27.6[c]	7.3	24.1[d]	26.8	25.3[c,d]	29.5
1.6	27.3[c]	6.4	27.4[c]	10.6	12.5[d]	14.0	18.8[d]	28.7
3.2	not tested		6.2[c]	24.3	4.0[d]	0.0	4.4[d]	47.3

[a]$\bar{Y}$ = mean.
[b]CV = coefficient of variation.
[c,d]Means within rows with the same letter are not significantly different (α = 0.05).

TABLE 3. Variability between four replicate tests in the effects of 28-d exposure to acridine on the numerical occurrence of the primiparous instar of Daphnia magna

Concentration (mg/L)	Primiparous instar							
	Test 1		Test 2		Test 3		Test 4	
	$\bar{Y}$[a]	CV[b]	$\bar{Y}$	CV	$\bar{Y}$	CV	$\bar{Y}$	CV
0.0 (control)	6.6[c]	14.2	8.4[d]	9.9	8.5[d]	13.0	8.7[d]	36.6
0.0 (methanol control)	9.9[d]	20.2	10.9[d]	11.3	10.7[d]	11.5	10.8[d]	37.5
0.2	6.9[e]	14.8	7.9[c]	5.7	8.0[c]	0.0	9.3[d]	27.6
0.4	7.1[c]	14.4	8.1[d]	5.5	8.6[d]	14.3	9.1[d]	28.3
0.8	8.4[c]	12.7	8.7[c]	13.5	11.2[d]	27.0	10.5[d]	37.3
1.6	16.1[d]	62.4	15.9[d]	75.9	-[c,f]		-[c,f]	
3.2	not tested		-[d,f]		-[d,f]		-[d,f]	

[a]$\bar{Y}$ = mean.
[b]CV = coefficient of variation.
[c,d,e]Means within rows with the same letter are not significantly different (α = 005).
[f]No eggs were produced.

TABLE 4. Variability between four replicate tests in the effects of 28-d exposure to acridine on the age of first brood release of Daphnia magna

Concentration (mg/L)	Age at first brood release (days)							
	Test 1		Test 2		Test 3		Test 4	
	$\bar{Y}$[a]	CV[b]	$\bar{Y}$	CV	$\bar{Y}$	CV	$\bar{Y}$	CV
0.0 (control)	8.6[c]	10.9	11.6[d]	7.2	11.6[d]	12.6	11.9[d]	11.7
0.0 (methanol control)	11.9[e]	16.8	13.5[a]	11.4	13.0[c]	8.9	15.1[d]	10.9
0.2	8.9[e]	11.5	9.9[c]	4.5	11.2[d]	5.5	11.8[d]	13.9
0.4	9.1[c]	11.2	11.0[d]	9.3	11.4[d]	7.2	11.7[d]	12.8
0.8	10.4[e]	10.3	12.2[c]	5.5	14.6[d]	16.6	13.9[d]	12.6
1.6	17.6[d]	15.7	11.9[d]	6.3	-[c,f]		-[c,f]	
3.2	not tested		-[d,f]		-[d,f]		-[d,f]	

[a] $\bar{Y}$ = mean.
[b] CV = coefficient of variation.
[c,d,e] Means within rows with the same letter are not significantly different ($\alpha = 0.05$).
[d] No broods were produced.
[f] No broods were produced.

TABLE 5. Variability between four replicate tests in the effects of 28-d exposure to acridine on brood production of Daphnia magna

Concentration (mg/L)	Number of broods produced per female							
	Test 1		Test 2		Test 3		Test 4	
	$\bar{Y}$[a]	CV[b]	$\bar{Y}$	CV	$\bar{Y}$	CV	$\bar{Y}$	CV
0.0 (control)	7.5[c]	6.8	5.5[d]	15.4	5.5[d]	12.6	5.8[d]	39.4
0.0 (methanol control)	6.9[c]	11.4	5.5[d]	27.4	5.4[d]	31.5	5.1[d]	37.0
0.2	8.1[c]	7.7	7.1[d]	13.4	5.4[e]	15.2	6.3[f]	28.8
0.4	7.9[c]	5.7	6.7[d]	10.1	5.7[f]	16.2	5.3[f]	34.7
0.8	7.4[c]	9.3	4.5[d]	22.7	3.9[d]	52.8	4.1[d]	48.1
1.6	1.7[c]	76.6	0.6[d]	136.8	0.0[e]	0.0	0.0[e]	0.0
3.2	not tested		0.0[c]	0.0	0.0[c]	0.0	0.0[c]	0.0

[a] $\bar{Y}$ = mean.
[b] CV = coefficient of variation.
[c,d,e,f] Means within rows with the same letter are not significantly different ($\alpha = 0.05$).

CANTON & ADEMA (1978) studied the reproducibility of a 21-d D. magna chronic toxicity test. They reported variations in inhibition of D. magna reproduction of 20% or greater in duplicate tests with six compounds; however, LRCTs appeared to be reproducible in most of their experiments. Our interpretation of the results of CANTON & ADEMA'S study is tentative since statistical analyses of their data were not provided.

In conclusion, two of six toxicity criteria, namely the number of young per brood and the young produced per female,

were found to be the most reliable and sensitive for estimating the LRCT for acridine to D. magna. Using either (or both) of these criteria, the D. magna static-renewal life-cycle toxicity test is a reproducible and sensitive test for estimating LRCTs. However, responses of individual toxicity criteria can be expected to vary significantly between tests done at different times.

TABLE 6. Variability between four replicate tests in the effects of 28-d exposure to acridine on brood size of Daphnia magna

Concentration (mg/L)	Number of young per brood							
	Test 1		Test 2		Test 3		Test 4	
	$\bar{Y}$[a]	CV[b]	$\bar{Y}$	CV	$\bar{Y}$	CV	$\bar{Y}$	CV
0.0 (control)	20.5[c,d]	10.9	18.1[d]	30.2	18.0[d]	32.6	22.2[c]	23.6
0.0 (methanol control)	19.1[c]	13.3	18.2[c]	24.8	14.3[d]	34.3	14.8[d]	21.3
0.2	22.4[d]	11.2	27.1[c]	20.4	15.7[e]	33.0	24.6[c,d]	16.8
0.4	23.5[d]	10.1	27.0[c]	14.1	14.4[e]	44.1	22.7[d]	27.8
0.8	16.3[c]	11.4	9.1[d]	27.3	8.8[d]	66.3	8.4[d]	40.1
1.6	2.4[c]	38.7	0.2[d]	203.5	0.0[e]	0.0	0.0[e]	0.0
3.2	not tested		0.0[c]	0.0	0.0[c]	0.0	0.0[c]	0.0

[a]$\bar{Y}$ = mean.
[b]CV = coefficient of variation.
[c,d,e]Means within rows with the same letter are not significantly different ($\alpha = 0.05$).

TABLE 7. Variability between four replicate tests in the effects of 28-d exposure to acridine on the production of young by Daphnia magna

Concentration (mg/L)	Number of young produced per female							
	Test 1		Test 2		Test 3		Test 4	
	$\bar{Y}$[a]	CV[b]	$\bar{Y}$	CV	$\bar{Y}$	CV	$\bar{Y}$	CV
0.0 (control)	153.2[c]	8.8	101.1[d]	41.2	98.7[d]	36.3	131.6[c]	46.4
0.0 (methanol control)	130.1[c]	7.7	103.7[d]	33.8	78.2[e]	46.2	75.3[e]	42.9
0.2	179.3[c,d]	9.3	189.7[c]	21.9	86.6[e]	39.7	155.1[d]	35.1
0.4	185.2[c]	11.1	180.5[c]	19.4	84.9[e]	45.1	120.8[d]	41.4
0.8	120.6[c]	11.9	42.7[d]	42.0	38.7[d]	82.9	35.6[d]	68.0
1.6	4.2[c]	84.3	0.2[d]	326.2	0.0[d]	0.0	0.0[d]	0.0
3.2	not tested		0.0[c]	0.0	0.0[c]	0.0	0.0[c]	
0.012								

[a]$\bar{Y}$ = mean.
[b]CV = coefficients of variation.
[c,d,e]Means within rows with the same letter are not significantly different ($\alpha = 0.05$).

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