United States
Environmental Protection
Agency

Office of Pesticides and
Toxic Substances
Washington, DC 20460

EPA 560/6-81-003
January 1981

Toxic Substances

EPA

Comparison of Static-Replacement and Flow-Through Bioassays Using Duckweed, Lemna gibba G-3

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COMPARISON OF STATIC-REPLACEMENT AND FLOW-THROUGH BIOASSAYS USING DUCKWEED, LEMNA GIBBA G-3

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ABSTRACT

Static-replacement and flow-through tests were conducted using $CuSO_4 \cdot 5H_2O$, 2,4,6-trichlorophenol, and o-cresol to determine if they gave similar LC50's and EC50's for duckweed, Lemna gibba G-3. Static-replacement tests also were conducted using ethylene glycol and di(2-ethylhexyl) phthalate. Mortality, reproduction, dry weight, and root length were used to measure effect levels of the toxicants. LC50's and EC50's were calculated using quadratic regression with log transformation of the independent variable (concentration) and with the following transformations for the dependent variables: arc sin square root of the proportion (p) of dead to total fronds on Day 7 (mortality), a log function yielding a growth rate constant K (reproduction), $\log_{10}$ dry weight and arc sin square root of the ratio of dry weight to control weight (dry weight), and $\log_{10}$ of root length. ANOVA's were used to test for differences between the two types of tests, tests within types, and replicates within tests. A procedure also was provided for estimating the number of tests and replicates necessary to obtain confidence limits within a given percentage of the mean.

Of the four effect parameters, mortality and reproduction produced the best results. The results generally indicated that the highest variation occurred among tests, regardless of type, and that the smallest variation was generally within tests (i.e. among replicates). Therefore, the conclusion was that the best allocation of resources would be to replicate static-replacement tests in time with the number of replicates dependent on the toxicant. Generally, four replicates should be used if no information is available on the expected variation within tests. The information gained can then be used to statistically determine the number of tests and replicates necessary to obtain given confidence limits and probability levels.

Key words: bioassay, duckweed, Lemna gibba G-3, aquatic toxicology, copper sulfate, 2,4,6-trichlorophenol, o-cresol, ethylene glycol, di(2-ethylhexyl)phthalate

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1.0 INTRODUCTION

The Office of Pesticides and Toxic Substances of the U.S. Environmental Protection Agency (EPA) is contemplating the development of a test standard, using duckweed as a test organism, for assessing the toxicity of certain wastes. To facilitate development of the protocol, information on the precision and design of static and flow-through bioassay tests was needed. Lemna gibba G-3 was chosen as the test organism because of its taxonomic stability and previous work on its physiology. Moreover, it is small, planktonic, easily cultured, and reproduces vegetatively, allowing development of a homogeneous clone.

The objectives of this study were

(1) to compare static-replacement bioassays with flow-through bioassays in terms of cost, precision, and comparability of LC50's and EC50's;

(2) to ascertain the optimum distribution of replicates and tests for achieving certain levels of significance; and

(3) to determine the LC50's and EC50's (based on dry weight, reproduction, and root length) for five compounds:

   a) copper sulfate ($CuSO_4$)
   b) 2,4,6-trichlorophenol (2,4,6-TCP)
   c) o-cresol
   d) ethylene glycol
   e) di(2-ethylhexyl)phthalate.

Several stages of testing were performed (Section 5.0). To compare static-replacement and flow-through test results, paired tests were run simultaneously using the toxicants $CuSO_4$, 2,4,6-TCP, and o-cresol. Additional concentrations were used in the static-replacement tests to obtain better estimates of the EC50's for growth and reproduction. Static-replacement tests were also performed using ethylene glycol and di(2-ethylhexyl)phthalate. These toxicants were not used in flow-through tests due to their extremely high threshold effect levels.

Due to time constraints, data for each of the four parameters for which the LC50's and EC50's were calculated had to be collected on the same set of tests. When the

LC50's and EC50's occurred at widely separated concentrations, both could not be calculated. When this occurred the LC50 was considered most important and the test concentrations were adjusted accordingly. Additional concentrations were added to some of the later static-replacement tests to allow estimation of both LC50's and EC50's from the same tests. This was not feasible with the flow-through tests because of costs and dilutor design constraints.

An appropriate statistical model and transformation for calculating LC50's and EC50's were selected based on the coefficients of determination, variances, and statistical probabilities. The various combinations of models and transformations used are outlined in Section 6.0. Selection of the best model and transformation was extremely important as all comparisons and design calculations were based on the estimated LC50's and EC50's and the variances associated with them. Once the data were analyzed, the results were used to predict the number of tests and replicates necessary to obtain confidence limits within a given percentage of the mean. The results of the analyses using each of the four effect parameters were evaluated to determine which of the four were most appropriate for measuring the effect levels of the toxicants.

2.0 SUMMARY

Of the four parameters measured - mortality, reproduction, dry weight, and root length - mortality and reproduction data gave the best results. All of the effect parameters were collected from the same tests due to time and budget constraints. Therefore, concentration ranges were not optimal for each of the effect parameters. Estimation of the LC50's was deemed most important, and the concentration ranges were adjusted to bracket the expected LC50.

The mortality data indicated a significant difference only between the two types of tests for $CuSO_4$ ($P = 0.03$). The two types of tests for 2,4,6-TCP and o-cresol gave almost identical EC50's (Table 2-1). This was unusual because 2,4,6-TCP and o-cresol are much more volatile than $CuSO_4$. Static-replacement bioassays on volatile compounds typically underestimate the LC50; flow-through tests theoretically give a better estimate because of the continual replacement of the toxicant. The LC50's generated were used to determine the distribution of variances among types, tests, and replicates and to predict the number of tests and replicates necessary to yield certain confidence limits and probability levels (Table 7-6).

No mortality data were generated for di(2-ethylhexyl) phthalate because range-finding tests indicated that concentrations near 200,000 mg/L would be necessary to calculate the LC50. Flow-through tests were not conducted for ethylene glycol because the concentrations needed to produce LC50's were in the 10,000 to 50,000 mg/L range and therefore not cost-effective.

Reproduction data gave the best results when analyzed using a growth rate constant (Equation 6-13). This transformation produced the highest R^2 values and thus the best estimate of the EC50. The difference in reproductive stages of duckweed fronds in each replicate (Section 8.1) caused more variation, and thus less precision, in the EC50's than in the LC50's. Reproduction rates were generally higher in flow-through tests than in static-replacement tests, possibly due to the continuous addition of nutrients or the agitation caused by periodic additions of the nutrients. No indication of crowding or nutrient depletion was observed in the static-replacement tests.

ANOVA's were used to test for reproduction rate differences between static-replacement and flow-through tests.

Table 2-1. Summary LC50 Data from Quadratic Regression Using Arc Sin Square Root Transformation for Paired Flow-through and Static-Replacement Tests.

Toxicant	Test Type	No. of Tests	$\bar{x}$	Range Min.	Range Max.	95% Confidence Limits		P*
$CuSO_4$	F & S	8	8.90	3.67	15.00	5.96	13.30	0.03
	F	4	6.76	3.67	10.46	3.27	13.93	
	S	4	11.71	7.03	15.00	6.59	20.80	
2,4,6-TCP	F & S	14	2.04	0.87	3.12	1.63	2.55	0.64
	F	7	2.04	0.87	3.12	1.45	2.87	
	S	7	2.03	0.93	3.11	1.35	3.05	
o-cresol	F & S	8	500.8	382.6	691.7	426.6	588.8	0.27
	F	4	464.3	382.6	577.7	346.7	616.6	
	S	4	540.3	436.9	691.7	398.1	741.3	
ethylene glycol	S	5	25760	17000	40140	16360	40560	

*P = probability of difference between types

Results indicated that the two types of tests yielded significantly different EC50's at or below the 0.11 probability level (Table 2-2). Predictions of the number of tests and replicates needed to obtain confidence limits within a given percentage of the mean EC50 (Table 8-4) were generally higher than those necessary to obtain LC50 confidence limits within the same percentage of the mean (Table 7-6).

The root length data could not be analyzed adequately because dead roots fell off the fronds and were too fragile to measure; thus data sets for individual replicates were incomplete, resulting in poor regressions and inaccurate estimations of EC50's based on root length. In many cases EC50's could not be estimated.

Toxicant concentration ranges were too high to yield meaningful EC50 data based on dry weight. The concentration ranges were established to produce the best LC50 estimates, and limited funding and time constraints prevented conducting tests designed to yield valid dry weight data. O-cresol tests yielded the only data which were complete enough to compare static-replacement and flow-through tests. The two types of tests were significantly different at the 0.11 probability level (Table 2-3).

Table 2-2. Summary EC50 Data from Quadratic Regression Using Growth Rate Constant Transformation for Paired Flow-through and Static-Replacement Tests.

Toxicant	Test Type	No. of Tests	$\bar{x}$	Range Min.	Range Max.	95% Confidence Limits		P*
$CuSO_4$	F & S	10	2.82	1.20	5.95	2.16	3.68	0.11
	F	5	2.21	1.20	2.65	1.76	2.79	
	S	5	3.51	2.92	5.95	2.16	5.70	
2,4,6-TCP	F & S	12	0.07	0.01	0.95	0.02	0.19	0.10
	F	6	0.03	0.03	0.09	0.01	0.05	
	S	6	0.13	0.03	0.95	0.02	0.90	
o-cresol	F & S	8	192.7	139.9	371.7	147.3	252.3	0.05
	F	4	151.5	139.9	154.6	140.9	161.9	
	S	4	245.8	190.5	371.7	155.7	388.0	
ethylene glycol	S	5	17159	12796	27179	12130	24274	
di(2-ethylhexyl) phthalate	S	7	2060	397	7582	706	6008	

*P = probability of difference between types

Table 2-3. Summary EC50 Data from Quadratic Regression Using Arc Sin Square Root of the Ratio Dry Weight to Control Weight Transformation for Paired Flow-through and Static-Replacement Tests.

Toxicant	Test Type	No. of Tests	$\bar{x}$	Range Min.	Range Max.	95% Confidence Limits		P*
o-cresol	F & S	8	23.9	0.17	127.6	7.73	73.79	0.11
	F	4	32.0	6.93	127.6	4.71	218.3	
	S	4	17.0	0.17	77.9	0.88	328.9	

*P = probability of difference between types

3.0 CONCLUSIONS

Static-replacement and flow-through tests yielded similar results for mortality data on 2,4,6-TCP and o-cresol. The mean LC50's for the two types of $CuSO_4$ tests were statistically different ($P = 0.03$). The majority of the variation within each toxicant occurred between tests. For all toxicants and groupings of static-replacement, flow-through, and combined static-replacement and flow-through tests, the between tests variation averaged 4X the within test variation. Thus, in order to obtain the best estimate of the true LC50, resources should be allocated to conduct several tests at different times. This should be done even at the expense of within test replication if necessary.

The variation between tests also was greater than between types of tests (static-replacement and flow-through). Therefore, if resources are limited, several replicated static-replacement tests should be conducted rather than one or a few flow-through tests.

Static-replacement and flow-through tests produced similar results for reproduction data on $CuSO_4$ and 2,4,6-TCP ($P = 0.11$ and $P = 0.10$, respectively). The EC50's generated by the two types of o-cresol tests were statistically different ($P = 0.05$). Variance components were not as evenly distributed in the reproduction data as in the mortality data. In static-replacement tests an average of 65% of the variation was between tests, whereas in flow-through tests the between test variation averaged 9% (Table 8-4). This indicates that the best allocation of resources for static-replacement tests would be to conduct several tests with only a few replicates (Table 8-4). On the other hand, flow-through tests should be highly replicated with fewer tests allocated across time; however, from a practical standpoint, this would be difficult to implement. Additionally, there is more variation among tests than between types, so that conducting several replicated static-replacement tests is the preferred alternative.

The dry weight data were poor for all toxicants except o-cresol. The o-cresol data were complete enough to allow comparison of static-replacement and flow-through tests. The two types of tests were not significantly different ($P = 0.11$). Partial correlation coefficients indicated that dry weight data did not contain a significant

amount of information beyond that contained in the mortality and reproduction data. Given these results, the variability of the data, and the high cost of collecting it, dry weight data collection is not recommended unless funds allow additional tests to be designed specifically for dry weight data collection. Dry weight did appear to be affected by much lower toxicant concentrations than did mortality or reproduction. Therefore, in appropriately designed tests, meaningful data probably could be obtained.

The root length data did not produce meaningful EC50's (Section 10.0). Fragmentation of the roots after death of the frond precluded their measurement.

4.0 RECOMMENDATIONS

Based on results of this study, the best allocation of resources would be to conduct static-replacement tests instead of flow-through tests. Mortality and reproduction (measured by the growth rate constant) should be assessed at the conclusion of seven-day tests. The tests should be replicated in time, with four replicates of each concentration and the control. A minimum of four tests should be conducted to allow sufficient estimation of the population LC50 or EC50 and calculation of test statistics.

Test chambers should be 250 ml beakers or larger and should contain at least 150 ml, or approximately 2 in. in a larger chamber, of a suitable growth medium such as Hillman's M-medium. Duckweed should be added as complete colonies; to prevent damage, fronds should not be separated. Three 4-frond colonies should be used when possible, although the data indicated no differences in LC50's and EC50's using only one 4-frond colony. The medium in each test chamber should be replaced at least every third day during the testing. Lighting should be continuous at 500 ± 50 fc.

The data should be analyzed by quadratic regression with log transformation of concentration, arc sin square root transformation of the proportion (p) of dead to total fronds, and log transformation of the reproduction data (Equation 6-13). An LC50 or EC50 should be calculated for each replicate and analyzed by an ANOVA to test for statistical differences between replicates. If differences are significant, the test data and experimental technique should be examined before continuing. Assuming no differences, a mean LC50 or EC50, standard error, confidence limits, and range should be calculated for each test.

The replicate LC50 or EC50 data should also be analyzed using a nested ANOVA to partition out the variance components between and within tests (Section 6.1.4). Variance components should be examined to determine the distribution of the variation. Normally, the largest variation occurs between tests, and a single mean LC50 or EC50, ranges, confidence limits, and standard errors should be calculated for the toxicant. The mean square and degrees of freedom for between tests should be used to calculate the confidence limits. If confidence limits are larger than desired, the mean square from the nested

ANOVA and Equations 6-11 and 6-12 should be used to determine how many additional tests should be conducted to narrow the confidence limits to within the desired range.

5.0 LABORATORY PROCEDURES AND METHODS

5.1. Selection of Test Species

Lemna gibba G-3 was chosen as the test species because of its taxonomic stability and previous research on its physiology. L. minor was not chosen because it cannot be definitively identified as a laboratory organism due to the absence of flowering (Hillman pers. comm. 1980). Furthermore, the literature does not deal with a specific strain of L. minor. L. gibba G-3 does flower and has been defined as a laboratory organism. It has been used extensively in research in the United States, Japan, and Europe. Axenic cultures of L. gibba G-3 were obtained from Dr. W.S. Hillman at the Brookhaven National Laboratory in New York.*

5.2. Selection of Toxicants

The EPA Office of Toxic Substances selected toxicants which are representative of a wide variety of compounds likely to be assayed in duckweed bioassays. The compounds varied from a soluble inorganic compound to insoluble organic compounds (Table 5-1). A log of tests conducted is presented in Table 5-2. Carriers were not used in the testing as they are not present in natural situations.

Range-finding tests were conducted to determine the "100%" concentration for each toxicant to be used in the definitive tests. Ten concentrations of sufficient range were used to determine the lowest concentration at which there was 100% mortality. Range-finding tests were conducted in 250 ml glass beakers for seven days. Equipment, chemicals, and procedures were the same as those used in the static-replacement tests (Section 5.5).

5.3. Culture Methods

Stock cultures of Lemna gibba G-3 were maintained in three to six 6 L glass aquaria covered with Nytex screen.

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Table 5-1. Test Toxicants and Their Physical Characteristics.

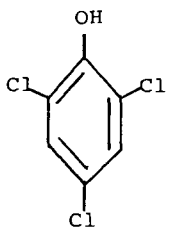
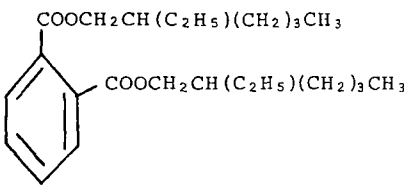
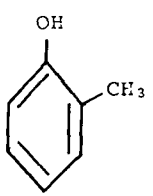
Toxicant	Formula	Solubility in H_2O	Use
copper sulfate	$CuSO_4 \cdot 5H_2O$	decomposes (18.2g/100g H_2O at 25°C)	algicide, fungicide, herbicide, bactericide
2,4,6-trichlorophenol		(0.09g/100g H_2O)	fungicide, bactericide, preservative
di(2-ethylhexyl) phthalate [bis(2-ethylhexyl) phthalate]		not soluble in water	vacuum pumps
ethylene glycol	$HOCH_2CH_2OH$	miscible with water	antifreeze, in hydraulic fluids, solvent in industrial processes
o-cresol (o-hydroxytoluene)		soluble in 40 parts water (2.5g/100g)	disinfectant, solvent

Table 5-2. Test Log.

Test #	Toxicant	Test Type[1]	Date
1-14		Trials, Range	6/25-8/23/79
15	ethylene glycol	Range	8/23/79
16	di(2-ethylhexyl)phthalate	Range	8/23/79
17	2,4,6-TCP	Range	8/23/79
18	2,4,6-TCP	Flow	8/23/79
19	2,4,6-TCP	Static	8/23/79
20	di(2-ethylhexyl)phthalate	Range	9/19/79
21	ethylene glycol	Range	9/19/79
22	$CuSO_4$	Range	9/19/79
23	ethylene glycol	Range	9/19/79
24	$CuSO_4$	Range	9/19/79
25	o-cresol	Range	9/19/79
26	2,4,6-TCP	Range	9/19/79
27-28		Range, Growth	9/19/79
29-31	(test numbers omitted)		
32	$CuSO_4$	Flow	9/27/79
33	$CuSO_4$	Static	9/27/79
34	di(2-ethylhexyl)phthalate	Range	9/28/79
35	2,4,6-TCP	Flow	10/11/79
36	2,4,6-TCP	Static	10/11/79
37	ethylene glycol	Static	10/12/79
38	o-cresol	Range	10/12/79
39	ethylene glycol	Static	10/23/79
40	di(2-ethylhexyl)phthalate	Static	10/23/79
41	di(2-ethylhexyl)phthalate	Static	10/23/79
42	o-cresol	Flow	11/01/79
43	o-cresol	Static	11/01/79
44	di(2-ethylhexyl)phthalate	Static	11/13/79
45	ethylene glycol	Static	11/13/79
46	$CuSO_4$	Flow	11/29/79
47	$CuSO_4$	Static	11/29/79
48	$CuSO_4$	Flow	12/13/79
49	$CuSO_4$	Static	12/13/79
50	ethylene glycol	Static	12/13/79
51	o-cresol	Flow	1/03/80
52	o-cresol	Static	1/03/80
53	di(2-ethylhexyl)phthalate	Static	1/03/80
54	2,4,6-TCP	Flow	1/17/80
55	2,4,6-TCP	Static	1/17/80
56	2,4,6-TCP	Static[2]	1/17/80
57	$CuSO_4$	Static	1/17/80
58	$CuSO_4$	Static[2]	1/17/80
59	di(2-ethylhexyl)phthalate	Range	1/25/80
60	o-cresol	Flow	1/31/80

Table 5-2 (Continued).

Test #	Toxicant	Test Type[1]	Date
61	o-cresol	Static	1/31/80
62	o-cresol	Static[2]	1/31/80
63	di(2-ethylhexyl)phthalate	Static	1/31/80
64	2,4,6-TCP	Flow	2/14/80
65	2,4,6-TCP	Static	2/14/80
66	ethylene glycol	Static	2/14/80
67	ethylene glycol	Static[2]	2/14/80
68-69		Reproduction	2/28/80
70	o-cresol	Flow	3/06/80
71	o-cresol	Static	3/06/80
72	$CuSO_4$	Static	3/11/80
73	$CuSO_4$	Static[2]	3/11/80
74	2,4,6-TCP	Flow	3/20/80
75	2,4,6-TCP	Static	3/20/80
76	2,4,6-TCP	Static	4/03/80
77	2,4,6-TCP	Static[2]	4/03/80
78	$CuSO_4$	Static	4/03/80
79	$CuSO_4$	Static[2]	4/03/80
80	2,4,6-TCP	Static	4/17/80
81	$CuSO_4$	Static	4/17/80
82-84	toxicant carrier	Range	5/01-5/08/80
85	$CuSO_4$	Flow	5/22/80
86	$CuSO_4$	Static	5/22/80
87	toxicant carrier	Range	5/22/80
88	2,4,6-TCP	Flow	6/05/80
89	2,4,6-TCP	Static	6/05/80
90	di(2-ethylhexyl)phthalate	Static	6/05/80
91	2,4,6-TCP	Flow[3]	6/19/80
92	2,4,6-TCP	Static	6/19/80
93	di(2-ethylhexyl)phthalate	Static	6/19/80
94	2,4,6-TCP	Flow	6/26/80
95	2,4,6-TCP	Static	6/26/80

[1] Flow = Flow-through
Static = Static-replacement
Range = Range-finding

[2] Modified -- only one 4-frond colony per beaker instead of three 4-frond colonies per beaker.

[3] Aborted

Lighting was adjusted to 500 ± 50 foot candles (fc) measured at the water surface. Cultures were thinned three times a week so that they covered approximately half the surface area of each aquarium. After thinning, the remaining organisms were agitated twice with Hillman's medium in a 500 ml Erlenmeyer flask to remove algae and poured over Nytex screen to drain off the suspended algae.

An axenic culture of duckweed was maintained at all times on nutrient medium and agar. Working stock cultures were established from this axenic reserve culture; however, working cultures were not maintained axenically. If axenic stock cultures were used then 1) the tests themselves should be conducted axenically, or 2) an intermediate acclimation step should be incorporated between the axenic cultures and the definitive tests. The first alternative, axenic flow-through tests, would be very costly to conduct because large volumes of nutrient solution and toxicants would require sterilization. Moreover, the entire dilutor system would have to be sterilized prior to each test. If contamination occurred during any stage of the test, all data would have to be discarded and a new test initiated after sterilization. Continuous lighting and the rapid reproductive rate would deplete the carbon dioxide in the container and consequently affect growth rates. Filtered carbon dioxide would have to be pumped into the chambers to assure an adequate supply. Also, nutrient solutions might be subtly altered by millipore filtering or autoclaving.

If organisms from axenic working stock cultures were used in definitive tests which were not conducted axenically, culture organisms would not be acclimated to test conditions. For example, the glass containers used to enclose axenic cultures would remove portions of the light spectrum which would be present during testing. A minimum of an additional 3 to 5 days would be needed for acclimation. Ten days would be better to allow the cultures to cycle through several generations and thereby eliminate effects of the axenic conditions. In summary, it is generally recommended that working stock cultures be maintained under conditions identical to those used for definitive tests.

5.4. Flow-through Tests

5.4.1. Equipment and Chemicals

Diluent and toxicant panels -- design. The dilutor system used was a modified EPA design (Peltier 1978). It consisted of a panel of diluent proportioning chambers and a panel of toxicant proportioning chambers, operated by a cycle control panel (Figure 5-1). Measured amounts of diluent and toxicant were drained synchronously into final

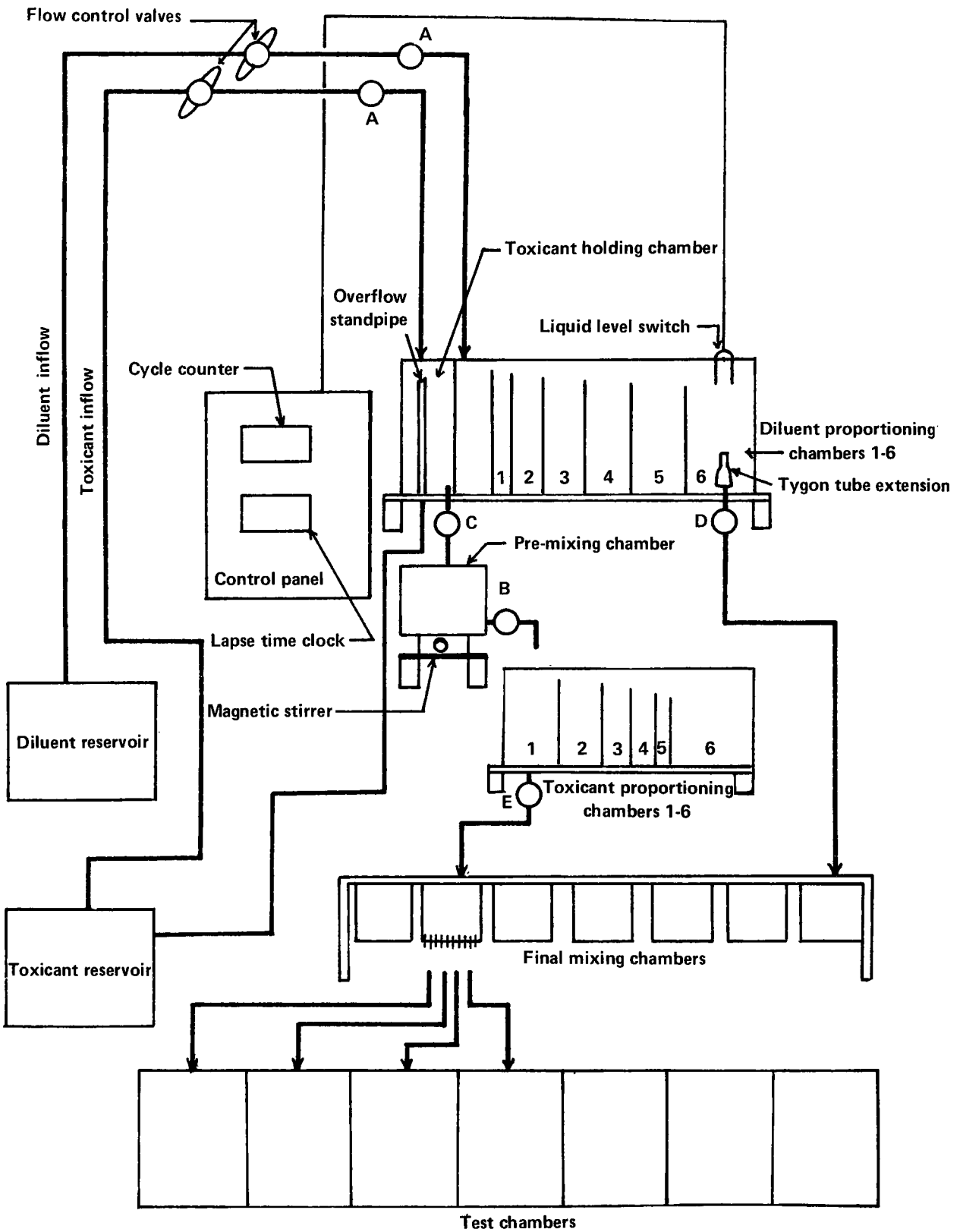


Figure 5-1. EPA-type flow-through dilutor system. A and B, normally open solenoid valves; C, D, and E, normally closed solenoid valves.

mixing chambers which emptied into test chambers containing the test organisms.

Two siphon-connected 240 L aquaria served as diluent reservoirs; one 240 L aquarium served as the toxicant reservoir. A submersible pump fed both diluent and toxicant to the control panel via Tygon lines (formula 3603, 3/8 in.). A submersible pump in the toxicant reservoir agitated the toxicant to keep it evenly mixed.

There were eight diluent proportioning chambers -- seven overflow-connected and one isolated chamber. Each of the seven connected chambers received a diluent (nutrient) solution from the reservoir via a line controlled by solenoid and variable flow valves. The solution level was controlled by a liquid level switch. Each chamber drained into a specific mixing chamber via a #316 stainless steel line (1/8 in. inside diameter) controlled by an in-line solenoid valve (Peter Paul model #7lP9ZGV).

The isolated chamber (toxicant holding chamber) in the diluent proportioning chamber panel received toxicant solution from the toxicant reservoir via a line also controlled by solenoid and variable flow valves. The solution level in the chamber was controlled by an overflow standpipe connected to the toxicant reservoir. The working solution (2216 ml) in this isolated chamber drained into the toxicant pre-mixing chamber via a stainless steel line controlled by a solenoid valve.

The toxicant pre-mixing chamber, mounted on a magnetic stirrer, emptied into six toxicant proportioning chambers via a Tygon tubing line controlled by a solenoid valve. The six chambers were overflow-connected; each drained into a specific final mixing chamber, along with a specified amount of diluent solution. One of the final mixing chambers received only diluent solution, and a second received only toxicant solution.

The volumes of solution delivered by the panels were controlled by Tygon tubing extensions on the drain lines in each chamber to insure precise dilution of the toxicant (Table 5-3).

Each final mixing chamber was drained by four Tygon lines (formula 3603, 1/4 in.); each of the four lines emptied into a 6 L glass aquarium serving as a test chamber for the test organisms. Therefore, each of the seven final mixing chambers drained into four aquaria. A Nalgene twist valve at the end of each Tygon line equalized flow in the four lines from each final mixing chamber. These valves corrected for the unequal flow rates in the lines created by different line lengths and surface tension. An elevated, sloped centerboard, painted with white epoxy paint, supported the Tygon lines feeding the aquaria.

Table 5-3. Volumes of Diluent and Toxicant Delivered by the Proportioning Chambers.*

Chamber	Volume (ml)	
	Diluent	Toxicant
1	440	560
2	680	320
3	820	180
4	900	100
5	944	56
6	1000	1000

*Like-number diluent and toxicant chambers mixed and emptied into a final 1000 ml mixing chamber (i.e., $D_1 + E_1 = 1000$ ml) with the exception of diluent and toxicant chambers 6, which emptied into separate final mixing chambers.

Each 6 L glass receiving aquarium (test chamber) had an overflow hole drilled at the 2 L level. A Nytex screen, cemented to the aquarium with silicone rubber, covered the hole. The aquaria were arranged in four rows, seven aquaria per row. Two galvanized tin trays, painted with white epoxy paint, each held two rows of seven aquaria. The sides of the holding trays were lined with aluminum foil for additional light scattering. Overflow from the aquaria drained into the trays and then into a galvanized tub containing a float-switched submersible pump (Little Giant model 1-M). Four-foot fluorescent light fixtures (Lakewood Engineering and Manufacturing Co.) with two light bulbs (Vita Lights) were suspended on chains above the aquaria to permit adjustable light settings.

Diluent and toxicant panels -- operating sequence. The flow apparatus was adjusted at the time control panel to cycle approximately once every 30 minutes. The final mixing chambers each received a 1000 ml solution of toxicant and diluent and subsequently delivered 250 ml to each of four aquaria. Thus the 250 ml of diluted toxicant emptying into each aquarium every half hour resulted in a complete volume change in each aquarium every four hours.

The time control panel activated the pumps in the diluent and toxicant reservoirs, opened solenoid valves A and B, and closed solenoid valves C, D, and E. Thus the pumps in the reservoirs filled the diluent proportioning chambers and the toxicant holding chamber, while the toxicant solution in the pre-mixing chamber drained into the toxicant proportioning chambers. Flow rates in the reservoir lines were adjusted to allow the toxicant holding chamber to fill before the diluent proportioning chambers were full. When the diluent solution reached the diluent liquid level switch, solenoid valves A and B were closed, the reservoir pumps were shut off, and solenoid valves C, D, and E were opened. This set of synchronous events allowed the diluent and toxicant proportioning chambers to drain into their respective final mixing chambers while the toxicant holding chamber refilled the pre-mixing chamber. The 1000 ml in each final mixing chamber subsequently drained into the designated test chambers.

Mixing of Hillman's medium. Hillman's M-medium was used as the nutrient solution for culturing the duckweed and as a diluent in the tests (Hillman 1961b). The solution was mixed in a 720 L stainless steel (#316) tank using analytical reagent grade chemicals. Calibrated glass bottles were used to measure deionized water into the tank. A specified volume of 678.53 L was standardly mixed to minimize error. Stock chemicals for adding to the tank were maintained in high but stable concentrations (Table 5-4). Micronutrients were premixed in a standard solution to minimize measurements of stock chemicals.

Table 5-4. Concentrations of Stock Chemicals Used to Mix Hillman's M-Medium.

Chemical	Concentration (g/L)
Potassium monobasic phosphate (KH_2PO_4)	200
Potassium nitrate (KNO_3)	250
Calcium nitrate [$Ca(NO_3)_2 \cdot 4H_2O$]	400
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	400
Ferric chloride ($FeCl_3$)	21.6
Tartaric acid [$HOOC(CHOH)_2COOH$]	12.0
Micronutrients	
Boric acid (H_3BO_3)	2.86
Zinc sulfate ($ZnSO_4$)	0.22
Sodium molybdate ($NaMoO_4$)	0.12
Cupric sulfate ($CuSO_4$)	0.08
Manganese chloride ($MnCl_2$)	3.62

After adding deionized water and stock chemicals to the tank, 10 N NaOH (sodium hydroxide) was added to raise the pH to a range of 4.8 to 5.2. A submersible pump (Little Giant model 1) circulated the solution in the tank to insure uniform concentration. After pH adjustment, the Hillman's solution (diluent) was pumped into the diluent reservoir via a Tygon line (formula 3603, 1/2 in.) and was measured into the toxicant reservoir with a calibrated bottle.

Toxicants. Each flow-through test was conducted with analytical reagent grade toxicants $CuSO_4 \cdot 5H_2O$, o-cresol, and 2,4,6-TCP. For each test, the toxicant was diluted by the following log scale percentages of the highest concentration selected: 100, 56, 32, 18, 10, 5.6, and 0.

5.4.2. Operating Procedures

Duration of tests. Before initiating flow-through tests, equipment was operated approximately 48 cycles without duckweed in the test chambers. These preliminary cycles allowed the test chambers to be filled with the proper toxicant dilutions and to equilibrate prior to introduction of the test organisms. Flow-through tests, with duckweed in the test chambers, were conducted for seven days (168 h = 336 cycles) for each toxicant at seven different dilutions.

Randomization of test chamber deliveries. The particular toxicant dilution received by each of the 28 test chambers was determined with a random numbers table. Rerandomization was performed prior to each new test.

Calibration of equipment. On Days 0, 4, and 7 equipment was checked for proper operation and recalibrated as necessary. Volume delivery of the diluent and toxicant panel chambers was adjusted with the use of Tygon tubing (1/4 in.) extended at variable lengths above the top of the standpipe within each chamber. The volume delivery to test chambers was calibrated by the addition of 1000 ml deionized water to each chamber and adjustment of Nalgene twist valves at the ends of the test chamber delivery tubes. Lights were adjusted to deliver 500 $\pm$ 50 fc measured at water surface level with a Protomatic photometer beneath the middle and ends of each light.

Hillman's medium and toxicant mixing schedules. Hillman's medium was mixed in the tank approximately every other day during the seven-day run. Usually enough medium was immediately pumped out to fill the one toxicant and two diluent reservoirs. The toxicants, mixed in Hillman's medium, were then added at the proper concentration to the toxicant reservoir.

Assessment of effects. Upon initiation of an experiment (Day 0), three approximately equal-sized, 4-frond duckweed colonies from the same stock culture aquarium were placed in each test chamber. Duckweed fronds and colonies were inspected on Days 1 (approximately 24 hours after test initiation), 4, 5, 6, and 7. Fronds were assessed for the following characteristics:

(1) Total number of fronds - number of fronds in each test chamber, without regard to possession of any of the following characteristics. New fronds budding from the brood pouch were counted as whole fronds.

(2) Necrotic fronds - fronds possessing localized regions of dead or decaying tissue, usually surrounded by healthy tissue. These regions may appear as gray, yellow, reddish, black and/or watery areas of obvious death and subsequent decay on an otherwise healthy frond.

(3) Chlorotic fronds - fronds possessing areas of progressive bleaching in color from green to yellow.

(4) Alive fronds - fronds that are either totally green or possessing necrotic and/or chlorotic characteristics.

(5) Dead fronds - fronds possessing no yellow or green tissue, usually all brown or white in color.

Colonies were inspected for the following characteristics:

(1) Total number of colonies - number of discrete groups of fronds without regard to characteristics of member fronds. Any solitary fronds were counted as one colony.

(2) Alive colonies - any colonies possessing one or more live fronds.

(3) Dead colonies - colonies possessing no live fronds.

Water quality measurement. Temperature, conductivity, and pH were measured in each test chamber on Days 0, 4, 5, 6, and 7 with a YSI 33 SCT conductivity/temperature meter (Yellow Springs Instruments Co.) and an Ionalyser model 407A pH meter (Orion Research). In addition, continuous measurements of temperature and conductivity were recorded in a randomly selected flow test chamber for the duration of each experiment.

Root length and weight measurement. Root length and weight of five 4-frond colonies from the original stock culture were measured on Day 0 before initiation of each test. On Day 7 after completion of an experiment, root length and weight of all duckweed in each test chamber were measured. Root lengths were measured with a ruler

to the nearest 1 mm. Weights were determined to the nearest 0.0001 g by drying the duckweed for 90 minutes in bottles over silica gel at room temperature at a negative pressure of 1 cm mercury (Blackman and Robertson-Cuningham 1953).

Equipment cleaning. All equipment was triple-washed with soap, hydrogen chloride (5% HCl), and acetone before reuse, as outlined in Peltier (1978). All Tygon tubing was replaced upon completion of each experiment.

5.5. Static-Replacement Tests

5.5.1. Equipment and Chemicals

Glass beakers (250 ml) served as test chambers for the static-replacement tests. Beakers were arranged in rows and placed on a table covered with a white vinyl cloth. Fluorescent light fixtures were suspended on chains above the beakers to permit adjustable light settings.

Static replacement tests were conducted with analytical reagent grade toxicants di(2-ethylhexyl)phthalate and ethylene glycol in addition to $CuSO_4 \cdot 5H_2O$, o-cresol, and 2,4,6-TCP. The Hillman's nutrient solution and the toxicant concentrations were the same as those used for flow-through tests (Section 5.4.1).

5.5.2. Operating Procedures

Duration of tests. Static-replacement tests were conducted for a total of seven days. All flow-through tests were run concurrently with a static-replacement test of the same toxicant.

Randomization of beakers. Arrangement of beakers was determined with a random numbers table. Rerandomization was performed prior to each new test.

Calibration of lights. Lights were adjusted to deliver 500 $\pm$ 50 fc measured at water surface level beneath the middle and ends of each light. On Days 4 and 7 light levels were checked and adjusted as necessary.

Toxicant mixing procedure. For static-replacement tests that were conducted concurrently with a flow-through test, the 100% toxicant concentration was obtained from the toxicant reservoir in the flow-through apparatus. The other six toxicant concentrations were obtained by sequentially diluting the solution from the toxicant reservoir with

Hillman's medium. The final toxicant concentrations obtained were the same as the seven toxicant concentrations delivered by the flow-through apparatus. Three additional concentrations were generally used to enhance the accuracy of EC50 predictions for reproduction and dry weight.

For static-replacement tests not conducted concurrently with a flow-through test, the sequence of toxicant concentrations was made by adding the toxicant to Hillman's medium obtained from the diluent reservoir in the flow-through apparatus. Once the maximum desired concentration ("100%") was obtained, this solution was then diluted as described above to yield the seven toxicant concentrations.

All beakers were filled to the 150 ml level with toxicant solution. Toxicant solutions were changed on Days 4 and 6 to control algal growth, and to prevent concentration of toxicants due to evaporative loss or dilution due to volatility. Duckweed colonies were lifted from each beaker with a small Nytex screen, cleaned of algae, and placed in a beaker with fresh solution.

Procedures for assessment of effects, water quality measurement, root length and weight measurement, and equipment cleaning were conducted as described for flow-through tests (Section 5.4.2).

5.6. Laboratory Conditions

Tests were conducted in a temperature-regulated room maintained at 25 $\pm$ 3°C.

5.7. Scheduling of Tests

Tests were scheduled with a 14-day (10 working days) interval between initiation of consecutive tests (Table 5-5). Each test was conducted for 7 days (Days 3-9, or 5 working days) followed by a 7-day period (5 working days) for breakdown and equipment cleaning, data compilation, and setting up the next test.

A 7-day test period, as opposed to a shorter period, was chosen for several reasons. Several days are needed for the fronds to adjust to the new experimental environment, less crowded conditions, flowing water, and handling (Hillman 1961a, Walbridge 1977). Toxic effects are more apparent over a longer period, particularly at low concentration levels. Growth rate differences are more apparent over a 7-day period because of the doubling rate of duckweed. Thus, the longer test period accentuates differences in growth and mortality.

Table 5-5. Outline of Tasks Performed During 14-Day Interval Between Tests.

Day #	Day of Week	Test Day #	Tasks
1	Tues.		Set up test; calibrate; mix chemicals; mix flow tanks.
2	Wed.		Set up test; mix chemicals; start flow apparatus.
3	Thurs.	0	Initiate test; measure water quality; select 4-frond colonies; obtain initial weights and root lengths; mix flow tanks.
4	Fri.	1	Make assessments; mix chemicals; fill flow tanks to last through weekend.
5	Sat.	2	None
6	Sun.	3	None
7	Mon.	4	Make assessments; measure water quality; mix chemicals; replace toxicants in static tests.
8	Tues.	5	Make assessments; measure water quality; mix chemicals.
9	Wed.	6	Make assessments; measure water quality; mix chemicals; replace toxicants in static tests.
10	Thurs.	7	Make assessments; measure water quality; measure dry weights and root lengths; break down test.
11	Fri.		Clean equipment.
12	Sat.		None
13	Sun.		None
14	Mon.		Clean equipment; set up for next test.

Scheduling tests to begin on Thursday (Table 5-5) leaves the weekends free of work, provided there is suitable storage capacity for the flow operation. A 5-day test period would still require a 14-day interval between tests to avoid any weekend work, i.e., 5 days of testing and 5 days of preparation and clean-up. The 7-day test period allows a more rigorous examination of toxic effects on duckweed without a real extension of the time period actually required for the tests.

6.0 STATISTICAL METHODS

6.1. Mortality Data Analysis

LC50's were calculated using several models and transformations to determine which combination of models and transformations gave the best estimate. The concentration of the toxicant was transformed using base ten logarithms. The proportion of dead fronds to the total number of fronds (p) was transformed using the following transformations:

angular: $y = \sin^{-1}\sqrt{p}$ (6-1)

logit: $y = G^{-1}(p)$, where G = logistic cdf (6-2)

normit: $y = F^{-1}(p)$, where F = normal cdf (6-3)

probit: $y = F^{-1}(p) + 5$, where F = normal cdf. (6-4)

Four types of models were used in the analysis:

linear: $y = \beta_0 + \beta_1 x + \varepsilon$ (6-5)

quadratic: $y = \beta_0 + \beta_1 x + \beta_2 x^2 + \varepsilon$ (6-6)

inverse: $x = \alpha_0 + \alpha_1 y + \varepsilon$ (6-7)

probit maximum likelihood: $E(p) = \Phi(\beta_0 + \beta_1 x)$ (6-8)

where β_0, β_1, and β_2, and α_0 and α_1 are regression coefficients, ε is the experimental error term, x is the $\log_{10}$ of concentration, y is the transformed value of p (p = proportion of dead fronds to total fronds), E(p) is the probability of killing a frond, and Φ is the normal distribution function.

6.1.1. Weighting Factors

Weighting coefficients were used in all calculations to account for dependence of the variance on the response rate. The general form of the weighting coefficients is given by Finney (1978). For the normit (or probit), logit, and angular (arc sin square root) transformations, the

weighting coefficients are

normit (or probit):	$\frac{(2\pi)^{-1}e^{-y^2}}{p(1-p)}$, where y = normit of p
logit:	$4p(1-p)$
angular:	4.

The weighting coefficients were incorporated by using them in a WEIGHT statement in SAS-GLM (SAS 1979). The combinations of models and transformations used are shown in Table 6-1.

6.1.2. Characteristics of Transformations

Angular. The angular transformation (6-1) is used to normalize percentages and proportions. It essentially makes the variance free of p which makes the basic assumptions of standard regression analysis more appropriate. Finney (1964) noted that, for all practical purposes, the angular transformation is a linear function of the probit transformation. One disadvantage is that the range over which the transformation is valid is finite. The model equation is invalid for very high and very low concentrations which produce near 0 or 100% mortality.

Logit. The logit transformation is $y = (1/2)\ln[p/(1-p)]$ (Finney 1978). The normit and logistic functions are very similar except in the extreme tails, and the resulting estimators will be similar. Berkson (1944, 1949) showed that the logit transformation could be essentially analogous to the probit. However, the logit transformation is much easier to compute.

Normit and probit. The normit transformation is the same as the probit transformation except that 5 is not added. Specifically, the normit is

$$y = F^{-1}(p), \qquad (6\text{-}3)$$

$$\text{where } F(x) = \int_{-\infty}^{x} (2\pi)^{-1/2} \exp(-t^2/2)\,dt$$

is the normal distribution function (Finney 1978). Both the probit and normit models assume a normal distribution of the lethal concentrations. The major disadvantage of these two transformations is that they are difficult to compute without the use of a computer and packaged routines such as SAS or BMD (Dixon 1974).

Table 6-1. Combinations of Models and Transformations Used to Analyze Mortality Data.

Model	Transformation	Procedure
Probit maximum likelihood (Finney 1964)	Probit	PROC PROBIT (SAS 1979)
Linear regression (Snedecor and Cochran 1967)	Arc sin square root Normit Logit	PROC GLM (SAS 1979)
Quadratic regression (Snedecor and Cochran 1967)	Arc sin square root Normit Logit	PROC GLM (SAS 1979)
Inverse regression (Shuster and Dietrich 1976)	Arc sin square root	PROC MATRIX (SAS 1979)

6.1.3. Models

Linear and quadratic regression models are commonly used statistical tools and do not require explanation (Snedecor and Cochran 1967). The inverse regression method is relatively new and requires a brief explanation. Its application to quantal response assays is discussed in detail by Shuster and Dietrich (1976). The procedure applies least squares estimation but reverses roles of independent and dependent variables. The model is

$$x = \alpha_0 + \alpha_1 y + \varepsilon \qquad (6\text{-}7)$$

where $x = \log_{10}(\text{concentration})$ and $y = \sin^{-1}\sqrt{p}$. The procedure uses weighted least squares, of the horizontal deviation from the regression line, to estimate model parameters. There are two major advantages of this model: 1) computations are straightforward and use standard algorithms, and 2) a confidence interval is always given and the interval is more "direct" because the parameter is estimated in the same scale as the deviation is minimized. Further, as stated by Shuster and Dietrich (1976), if the true model is not linear, the asymptotic variance is less than for other procedures. However, if the true model is not linear, then procedures that assume a linear model will produce biased LC50 estimates, i.e. the expected value of the LC50 estimate will not equal the true LC50 value. Thus, the estimates based on linear models will consistently either underestimate or overestimate the true LC50. For the duckweed experiments analyzed, the LC50 estimates obtained from the inverse regression procedure were lower than LC50 estimates based on other procedures, indicating a larger bias in the inverse regression estimates than in the other estimates. This bias negates the benefit of the smaller variance of the inverse regression procedure, and suggests a major disadvantage of the inverse regression procedure.

Maximum likelihood estimation is also a well-known procedure which has long been used in bioassay work and requires no explanation. A primary disadvantage is that the estimates often require an iterative solution. Finney (1977) gives a detailed discussion of maximum likelihood estimation using probit.

6.1.4. Variation of Mortality Data

ANOVA tests using SAS PROC GLM procedures were used to partition out sources of variation in the estimates (SAS 1979). LC50 estimates were computed by quadratic regression for each replicate of each test. The

transformed p values were then subjected to an ANOVA to test for replicate effects, goodness of fit of the linear regression models, and interaction between replicates and model terms. A table was computed for each of the three transformations. Table 6-2 is provided as an example.

To estimate the distribution of the variability of LC50 values split among tests and within tests, a nested analysis of variance was used (PROC NESTED, SAS 1979). The partitioned variance was then used to compute the number of tests and number of replicates per test to be used for future studies using similar compounds. Using the nested analysis, estimates were obtained for σ^2 and τ^2, where σ^2 is the variance of a log LC50 estimate based on one test with one replicate, and τ^2 is the variance component between tests. The variance (v) of the average log LC50 estimate with k tests and r replicates (Snedecor and Cochran 1967) is

$$v = \frac{\sigma^2 + r\tau^2}{kr} . \qquad (6\text{-}9)$$

To determine the optimum design, k and r must be selected to minimize v over the feasible values of k and r. The experimenter must decide within what percentage he must know the mean log LC50. Once these factors are known, the equation

$$t\sqrt{\frac{s^2 + rT^2}{kr}} < gm \qquad (6\text{-}10)$$

can be solved to give an estimate of the number of tests and replicates within tests which are needed to reach a certain level of precision. For Equation 6-10,

t = tabulated t value from standard tables with k-1 degrees of freedom
g = percentage within which the LC50 must be known
m = estimate of μ, the average log LC50
s^2 = estimate of σ^2, the within test variance
T^2 = estimate of τ^2, the among tests variance component
k = number of tests
r = number of replicates per test.

The values of m, s^2, and T^2 are obtained by conducting a nested analysis of variance of the form

Source of Variation	Expected Mean Square
Between Tests	$\sigma^2 + k_1 \tau^2$
Reps within Tests	σ^2

Table 6-2. Sample ANOVA to Determine Replicate Effects, Goodness of Fit of the Linear Regression Models, and Interaction between Replicates and Model Terms.

Dependent Variable:* arc sin square root of total fronds Day 7

Source	df	Sum of Squares	Mean Square
Model	11	159.28222214	14.48020201
Error	12	24.49222807	2.04101901
Corrected Total	23	183.77445021	

Source	df	Type I SS	F Value	PR > F
Rep. No.	3	2.57144157	0.42	0.7420
$\log_{10}$conc	1	130.85570857	64.11	0.0001
$\log_{10}$conc · $\log_{10}$conc	1	21.96578577	10.76	0.0066
$\log_{10}$conc · rep. no.	3	2.34475001	0.38	0.7672
$\log_{10}$conc · $\log_{10}$conc · rep. no.	3	1.54453622	0.25	0.8582

*weighted: 4 x total fronds Day 7

The quantity s^2 is the mean square for replicates within tests. The quantity T^2 is computed as T^2 =

$$\frac{\text{(between tests mean square) - (reps within tests mean square)}}{k_1}.$$

The value of k_1 is equal to a weighted average of the number of replicates in the tests and should not be confused with k or r in Equation 6-10.

Equation 6-10 can be rearranged algebraically to calculate the number of tests (k) and the number of replicates (r) within tests that are necessary to compress the confidence limits to within a given percentage (g) of the mean LC50. The minimum number of tests is calculated by

$$\frac{k}{t^2} > \frac{T^2}{(gm)^2} . \qquad (6\text{-}11)$$

The right-hand side of the equation is solved using estimates of T^2, g, and m from previous experiments using similar compounds or, if available, from tests using the same compound. Because k and t^2 are interrelated, a table relating them must be generated from standard t-tables. Once the minimum number of tests is known, the minimum number of replicates is computed using the formula

$$r > \frac{s^2}{(\frac{gm}{t})^2 k - T^2} . \qquad (6\text{-}12)$$

6.2. Reproduction Data Analysis

Reproduction, expressed as total number of fronds, was evaluated using two methods. The first method used quadratic regression (6-6) with a square root transformation of total frond number and a $\log_{10}$ transformation of the concentration. The square root transformation makes the variances independent of the means and generally is used to transform count data. A 50% reduction in total mean number of control fronds was chosen as the EC50.

The second method also used quadratic regression, but was based on a growth rate constant K:

$$K = \frac{\log_{10}(F_d) - \log_{10}(F_0)}{d} \qquad (6\text{-}13)$$

where F_d = number of fronds on Day d

F_0 = number of fronds on Day 0

d = day of assessment.

The EC50 was defined as the concentration which causes the growth rate constant to equal half the control (0 concentration) growth rate.

One drawback of using a quadratic regression is that confidence limits about a single EC50 estimate cannot be directly computed. However, they can be generated by having the computer calculate the confidence band about the regression line and projecting down from the points on the bands whose ordinates are the square root of half the average number of total fronds in the four replicates.

6.3. Dry Weight Data Analysis

Dry weight was analyzed by two methods. In the first, quadratic regression with a $\log_{10}$ transformation of concentration and dry weight was used to determine the EC50. Half the mean control (0 concentration) dry weight was selected as the effect level. This level was substituted into the equation for the regression line, and the log concentration was extracted by solving the equation. In the second method, quadratic regression was also used with a $\log_{10}$ transformation of the concentration, but the dependent variable, dry weight, was transformed using the arc sin square root of the proportion of dry weight to the mean control dry weight. The EC50 was calculated by substituting the arc sin square root of 0.5 (50%) into the regression equation and solving for the log concentration.

6.4. Root Length Data Analysis

The root length data were analyzed using linear and quadratic models with a $\log_{10}$ transformation of concentrations and root lengths. The coefficient of determination values were examined to determine if linear or curvilinear regression was appropriate for data analysis.

6.5. Comparative Methods

Partial correlation coefficients were used to determine if a dependent variable contained information about the effects of the toxicant in addition to the information contained in another variable. PROC GLM with MANOVA was

used to calculate the partial correlation coefficients (SAS 1979). The coefficients were computed according to the formula

$$r_{y_1 y_2 \cdot y_3} = \frac{r_{y_1 y_2} - (r_{y_1 y_3})(r_{y_2 y_3})}{(1-r^2_{y_1 y_3})(1-r^2_{y_2 y_3})} \qquad (6\text{-}14)$$

where y_1 and y_2 are the variables for which the correlation is being made while y_3 is held constant.

If $r_{y_1 y_2 \cdot y_3}$ is near 0, then y_2 contains no information about the effects of y_1 in addition to the information contained in y_3. Conversely, as $r_{y_1 y_2 \cdot y_3}$ approaches 1, y_2 increases in information about the effects of y_1 in addition to the information provided by y_3 about y_1. Thus $r_{y_1 y_2 \cdot y_3}$ measures the effects of y_1 on y_2 that are unrelated to the effects of y_1 on y_3. A value of $r_{y_1 y_2 \cdot y_3}$ near 1 would indicate that collection of data relating to y_2 would provide additional information beyond that contained in y_3.

7.0 MORTALITY DATA ANALYSIS

Coefficients of determination were used to determine if transformation of the mortality data could linearize the data. Examination of data such as that in Table 7-1 indicated linearization was not possible in the majority of the cases. ANOVA's were also computed to determine if the quadratic term in the regression model was significant. ANOVA tables similar to Table 7-2 were computed for each toxicant. The data indicated that the quadratic model with an arc sin square root transformation of the proportion of dead to total fronds was the most appropriate combination for calculation of the LC50's. The ANOVA procedure was also used to ascertain if significant differences occurred among replicates. Examination of the data indicated no significant interaction even at modest probability levels for the majority of tests (Table 7-2). Thus the replicates could be pooled for some data comparisons.

Although the quadratic model gave the best fit for the data, one drawback is that confidence limits cannot be calculated directly. However, the computer can be used to generate predicted values for the upper and lower confidence limits for selected concentrations. The confidence limits are then scanned for the value which would give the LC50. Because some experimenters might not have the facilities or the desire to make the necessary calculations, probit maximum likelihood computations were also made. As demonstrated for $CuSO_4$ (Figure 7-1), the probit method generally agreed with the quadratic method in estimation of the LC50. However, the confidence limits for the procedures showed less agreement. The inverse procedure generally gave much narrower confidence limits, but the procedure is biased because of incorrect assumptions (Section 6.1.3.).

In 89% of the tests analyzed by probit maximum likelihood, heterogeneity factors (h) were used to adjust the variances in calculation of the confidence limits. The heterogeneity factor (h) (Finney 1977) is designed to adjust for heterogeneous responses in the data and is calculated as

$$h = \chi^2/df \qquad (7\text{-}1)$$

where χ^2 = the Chi-square value and df = degrees of freedom calculated as (k-2) where k is the number of concentrations in the test. The h factor is used when a significant χ^2 value occurs for the test. A large χ^2 value can result from heterogeneity in the organisms or because the mathematical model used to fit the data is incorrect. The

Table 7-1. Coefficients of Determination from Linear and Quadratic Regressions on Mortality Data Using Arc Sin Square Root, Probit, and Logit Transformations for 2,4,6-TCP.

Test No.	Linear Regression			Quadratic Regression		
	Arc Sin $\sqrt{\ }$*	Probit*	Logit*	Arc Sin $\sqrt{\ }$*	Probit*	Logit*
18	0.505	0.307	0.213	0.649	0.561	0.509
19	0.768	0.656	0.551	0.775	0.742	0.712
35	0.301	0.133	0.090	0.640	0.392	0.291
36	0.230	0.129	0.093	0.550	0.416	0.356
54	0.532	0.299	0.188	0.708	0.544	0.403
55	0.564	0.361	0.270	0.790	0.618	0.496
64	0.607	0.538	0.500	0.769	0.709	0.663
65	0.520	0.406	0.334	0.734	0.692	0.649
74	0.704	0.523	0.415	0.785	0.657	0.557
75	0.376	0.178	0.105	0.648	0.444	0.333
76	0.344	0.219	0.170	0.629	0.517	0.456
80	0.277	0.096	0.042	0.470	0.181	0.070
88	0.776	0.702	0.648	0.835	0.795	0.765
89	0.349	0.161	0.106	0.721	0.482	0.350
92	0.478	0.269	0.188	0.762	0.642	0.549
94	0.616	0.495	0.452	0.821	0.707	0.643
95	0.526	0.392	0.326	0.759	0.642	0.568

*transformations

Table 7-2. ANOVA Results Using Arc Sin Square Root Transformation of $CuSO_4$ Mortality Data.

Test No.	MSE	R^2	PF-R	PF-L	PF-LL	PF-LR	PF-LLR
13	2.041	0.867	0.7420	0.0001	0.0066	0.7672	0.8582
14	1.682	0.868	0.8309	0.0001	0.0033	0.3722	0.2712
32	1.366	0.965	0.0015	0.0001	0.9351	0.2870	0.5794
33	1.285	0.961	0.4208	0.0001	0.0006	0.5958	0.1933
46	1.660	0.949	0.0069	0.0001	0.6896	0.5059	0.6228
47	2.201	0.902	0.3273	0.0001	0.0067	0.4791	0.6242
48	2.318	0.919	0.9550	0.0001	0.0106	0.6103	0.8762
49	1.511	0.939	0.8008	0.0001	0.0004	0.8530	0.8791
57	0.874	0.976	0.8063	0.0001	0.0534	0.3805	0.1307
58	0.664	0.926	0.0922	0.0001	0.0001	0.8244	0.3829
72	0.606	0.968	0.2022	0.0001	0.0004	0.3840	0.4495
73	0.403	0.915	0.0725	0.0001	0.0046	0.2514	0.9241
78	1.450	0.953	0.8199	0.0001	0.0007	0.9871	0.8728
79	0.567	0.928	0.3554	0.0001	0.0125	0.8853	0.3525
81	1.419	0.948	0.6681	0.0001	0.8390	0.4914	0.5287

MSE = mean standard error (within test error)

R^2 = $1 - \frac{\text{residual sum of squares from fitted model}}{\text{corrected total sum of squares}}$

PF-R = probability of obtaining a greater F if replicates are not different

PF-L = probability of obtaining a greater F if no linear effect of concentration (no overall increase or decrease trend)

PF-LL = probability of obtaining a greater F if no quadratic effect of concentration

PF-LR = probability of obtaining a greater F if no linear effect interaction with replicate

PF-LLR = probability of obtaining a greater F if no quadratic effect interaction with replicate

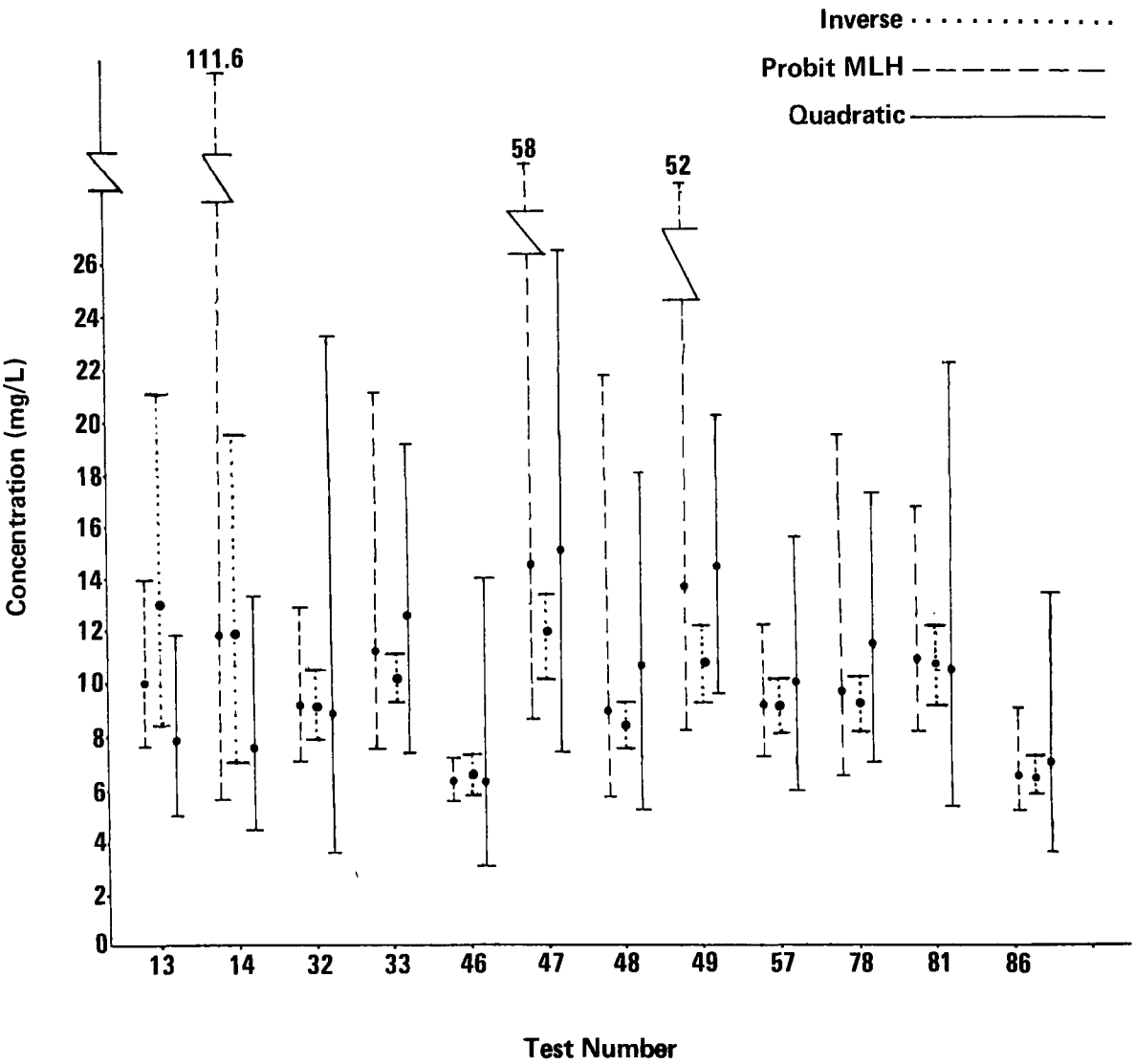


Figure 7-1. $CuSO_4$ LC50's and confidence limits using inverse, probit maximum likelihood (MLH), and quadratic regression with arc sin square root transformation of dependent variables.

duckweed data appear to suffer from the latter cause which is not corrected by use of the h factor. However, the computer program cannot distinguish the cause of the large χ^2 values, so it automatically calls the subroutine to apply the h factor. The h factor extends the confidence limits and thus makes the limits closer to what they should be. However, the h factor does not correct for the lack of model fit and should not be used to do so. The use of the h factor does not cause consistent over- or underestimation of the confidence limits (Figure 7-1). Little if any faith should be placed in confidence intervals calculated in this manner. The heterogeneity factor would have been eliminated in the analysis, but the SAS PROC PROBIT procedure used does not allow for overriding the heterogeneity subroutine (SAS 1979).

7.1. $CuSO_4$

All $CuSO_4$ results are based on the mg/L of $CuSO_4$ added as $CuSO_4 \cdot 5H_2O$. The approximate $CuSO_4$ toxicity can be estimated by multiplying the LC50 by 0.64 which is the percentage of $CuSO_4$ in $CuSO_4 \cdot 5H_2O$. The approximate Cu^{++} toxicity can be estimated by multiplying by 0.25. Concentrations used in the paired tests were 1.68, 3.0, 5.4, 9.6, 16.8, and 30.0 mg/L $CuSO_4 \cdot 5H_2O$.

Paired test results yielded mean LC50's of 6.76 mg/L and 11.71 mg/L for flow-through and static-replacement tests, respectively (Table 7-3). An ANOVA indicated that differences between LC50's for static-replacement and flow-through tests were significant at the 0.03 probability level (Table 7-4). Flow-through test LC50's varied slightly more than static LC50's. Coefficients of variation were 0.10 and 0.06 for flow-through and static-replacement tests, respectively. However, in both types of tests, the within test and between tests variation was proportioned similarly (Table 7-5). Approximately 84% of the variation occurred between tests.

LC50's for flow-through tests ranged from 3.67 to 10.46 mg/L (Table 7-3). The 95% confidence interval for the mean of the flow-through test LC50's ranged from 3.27 to 13.96 mg/L. These were within 38% of the mean LC50 (6.76 mg/L) in the log scale. The LC50's in the absolute scale are unsymmetrical because they were transformed from the log scale. The confidence interval must be calculated in the log scale, because it is incorrect to convert variances calculated for transformed data back to the absolute scale (Snedecor and Cochran 1967).

Static-replacement test LC50's ranged from 7.03 to 15.0 mg/L (Table 7-3). The 95% confidence interval ranged

Table 7-3. LC50's from Weighted Quadratic Regression with Arc Sin Square Root Transformation of Proportion of Dead Fronds to Total Fronds, Day 7.

Toxicant	Test No. and Type[1]		LC50	LCL[2]	UCL[3]
$CuSO_4$	32	F	8.84	3.60	23.08
	33	S	12.49	7.34	19.14
	46	F	6.25	2.98	13.91
	47	S	15.00	7.27	26.48
	48	F	10.46	5.07	17.99
	49	S	14.28	9.54	20.11
	85	F	3.67	2.17	7.34
	86	S	7.03	3.50	13.31
2,4,6-TCP	18	F	0.87	-	3.62
	19	S	0.93	-	3.18
	35	F	1.85	-	3.83
	36	S	2.01	-	-
	54	F	1.68	-	4.02
	55	S	1.45	-	3.27
	64	F	2.75	0.64	5.31
	65	S	2.92	0.61	5.80
	74	F	2.02	-	4.45
	75	S	2.04	-	4.58
	88	F	2.18	0.69	4.43
	89	S	2.85	-	5.21
	94	F	3.12	1.41	5.25
	95	S	3.11	1.10	5.62
o-cresol	42	F	495.44	232.56	851.83
	43	S	498.05	228.49	865.05
	51	F	577.65	304.75	-
	52	S	562.00	311.66	-

Table 7-3 (Continued).

Toxicant	Test No. and Type[1]		LC50	LCL[2]	UCL[3]
	60	F	440.33	254.01	653.79
	61	S	691.74	329.49	-
	70	F	382.60	217.18	563.64
	71	S	436.87	246.85	-
ethylene glycol[4]	37	S	17006	3099	48016
	39	S	29794	16602	49353
	45	S	28287	9935	-
	50	S	40139	26405	-
	66	S	21184	1948	-

[1]paired tests conducted during the same time period
F = flow-through test
S = static-replacement test

[2]LCL = lower confidence limit

[3]UCL = upper confidence limit

[4]no flow-through tests conducted

Table 7-4. ANOVA Table Comparing $CuSO_4$ Flow-through and Static-Replacement Paired Tests Conducted During the Same Time Period. Data entered were log_{10} LC50's generated by quadratic regression with arc sin square root transformation.

Dependent Variable: log_{10} LC50

Source	df	Sum of Squares	Mean Square
Model	7	1.22101261	0.17443037
Error	24	0.13997257	0.00583219
Corrected Total	31	1.36098518	

Source	df	Type I SS	F Value	PR > F
Type	1	0.45655418	78.28	0.0001
Date	3	0.67673112	38.68	0.0001
Type·Date	3	0.08772731	5.01	0.0077

Tests of Hypotheses Using the Type IV MS for Type·Date as an Error Term

Source	df	Type IV SS	F Value	PR > F
Type	1	0.45655418	15.61	0.0289
Date	3	0.67673112	7.71	0.0637

Table 7-5. Nested ANOVA to Determine Between Tests and Within Test Variation for $CuSO_4$ Tests. Data entered were log_{10} LC50's generated by quadratic regression with arc sin square root transformation.

Paired Flow-through and Static-Replacement Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	31	1.36099	0.04390	0.04798	100.00
Test No.	7	1.22101	0.17443	0.04215	87.84
Error	24	0.13997	0.00583	0.00583	12.16
Mean		0.949164			
Standard Deviation		0.076369			
Coefficient of Variation		0.080459			

Flow-through Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	15	0.55972	0.03731	0.04476	100.00
Test No.	3	0.46940	0.15647	0.03723	83.19
Error	12	0.09032	0.00753	0.00753	16.81
Mean		0.829718			
Standard Deviation		0.086756			
Coefficient of Variation		0.104561			

Static-Replacement Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	15	0.34471	0.02298	0.02769	100.00
Test No.	3	0.29506	0.09835	0.02355	85.06
Error	12	0.04965	0.00414	0.00414	14.94
Mean		1.068610			
Standard Deviation		0.064326			
Coefficient of Variation		0.060196			

from 6.59 to 20.80 mg/L, which is within 23% of the mean LC50. The static-replacement test LC50's were generally higher than the flow-through test LC50's. The contract did not allow research into the causes for this phenomenon. A possible explanation is that the fronds in the static-replacement tests were able to absorb and sequester a portion of the Cu^{++}. The reproductive rate of L. gibba results in a doubling of the number of fronds every two days. The new fronds may be able to absorb a portion of the replaced Cu^{++} which was changed every three days. Hutchinson (1975) stated that L. minor from natural waters in New Jersey which contained 0.009 mg/L Cu^{++} contained 32.5 ppm Cu^{++}. Thus plants apparently are able to take up and store some Cu^{++} without a detrimental effect. If this sequestering of the Cu^{++} occurred in the bioassays, the actual concentration of Cu^{++} in solution would decrease. This was not verified because no chemical measurements of toxicant concentration were allowed under this contract.

The data and Equations 6-11 and 6-12 were used to calculate the number of tests and replicates necessary to obtain confidence limits within a given percentage of the mean in log units (Table 7-6). Given the fact that the ANOVA indicated a significant difference between types (Table 7-4), separate predictions for flow-through and static-replacement tests probably should be used. However, given the closeness of the projections, the combined projection may be adequate (Table 7-6). The projection for obtaining confidence limits within 25% would be most appropriate given the magnitude of the mean and the costs of conducting the additional tests and replicates required to narrow the limits to within 10% of the mean LC50 (Table 7-6).

$CuSO_4$ was also used to assess the effect of fewer fronds per test chamber in three sets of paired static-replacement tests. One test in each pair contained 4 fronds per test chamber (Type I) and the other test contained 12 fronds per chamber (Type II). The Type I and Type II LC50's were 7.62 and 6.84 mg/L, respectively. An ANOVA indicated a significant difference in these LC50's at the 0.55 probability level. Thus based on $CuSO_4$ tests, reducing the number of organisms from 12 fronds to 4 fronds would not cause a loss in information. However, such a reduction would not necessarily result in a significant cost savings as the number of test chambers, amount of media, and labor needed to conduct the tests would be the same, regardless of the number of organisms used.

Table 7-6. Prediction of Minimum Number of Tests and Replicates Necessary to Obtain Confidence Limits Within 10%, 25%, and 50% of Mean at a 95% Probability Level (using $\log_{10}$ LC50's from quadratic regression with arc sin square root transformation for paired flow-through and static-replacement tests only).

Toxicant	Test Type	k 10%	r 10%	k 25%	r 25%	k 50%	r 50%	T^2	s^2	$\bar{x}$
$CuSO_4$	F & S	21	5	6	1	4	1	0.04215	0.00583	0.95
	F	24	6	6	5	4	1	0.03723	0.00753	0.83
	S	11	3	4	1	3	1	0.02355	0.00414	1.07
2,4,6-TCP	F & S	~120	5	21	6	7	19	0.02741	0.00794	0.31
	F	~105	11	19	7	7	3	0.02484	0.00738	0.31
	S	~150	5	26	5	9	2	0.03472	0.00848	0.31
o-cresol	F & S	3	1	3	1	2	1	0.00666	0.00124	2.70
	F	3	1	3	1	2	1	0.00610	0.00116	2.67
	S	3	1	3	1	2	1	0.00665	0.00133	2.73
ethylene glycol	S	3	2	3	1	2	1	0.02369	0.01121	4.41

F = flow-through test
S = static-replacement test
k = number of tests
r = number of replicates

T^2 = estimation of T^2 which is between test variation
s^2 = estimation of σ^2 which is between replicates within tests variation
$\bar{x}$ = mean $\log_{10}$ (LC50)

7.2. 2,4,6-TCP

Concentrations for paired definitive flow-through and static-replacement tests were 0, 0.34, 0.6, 1.10, 1.92, 3.36, and 6.0 mg/L. Additional concentrations were added to some static tests, but were not used in comparing static-replacement and flow-through tests. The paired static-replacement and flow-through tests had mean LC50's of 2.03 and 2.04 mg/L, respectively; an ANOVA indicated a statistical difference between these numbers at the 0.64 probability level (Table 7-7). Given the high volatility of TCP, a greater difference in static-replacement and flow-through tests would be expected. The contract did not allow for research into the reason for this lack of difference. Static-replacement test LC50's ranged from a low of 0.93 mg/L to a high of 3.11 mg/L TCP, while flow-through test LC50's ranged from 0.87 to 3.12 mg/L TCP (Table 7-3). The 95% confidence intervals for static-replacement and flow-through tests were 1.36 to 3.08 mg/L and 1.45 to 2.88 mg/L, respectively. Actual LC50's calculated for both types of tests exceeded these confidence limits (Table 7-3).

Although nested ANOVA's (Table 7-8) were calculated separately for static-replacement and flow-through tests, the combined nested ANOVA should be used to assess the distribution of variance components because 1) an ANOVA indicated no differences between LC50's for flow-through and static-replacement tests (Table 7-7), and 2) the combined data yield more degrees of freedom. The combined data indicated 78% of the variation occurred among tests as opposed to 22% within tests (Table 7-8). The actual LC50's support this breakout (Table 7-3). The LC50's for paired tests are much closer together than the LC50's for tests conducted at different times. The nested ANOVA (Table 7-8) indicates that the between tests variation is approximately 3X the within test variation.

Equations 6-11 and 6-12 and data from the nested ANOVA indicated the need for 21 tests with 6 replicates to obtain 95% confidence limits within 25% of the mean LC50 (Table 7-6). The confidence limits calculated using 14 tests and 4 replicates were within 31% of the mean LC50. Given the magnitude of the mean LC50 (2.0 mg/L), confidence limits within 50% of the mean should be acceptable. To obtain these confidence limits, 7 tests with 19 replicates would be needed (Table 7-6). It is more cost-efficient in terms of time and labor to conduct 7 tests with 19 replicates than to conduct 21 tests with 6 replicates because the tests need to be conducted at different points in time. Between tests variability is a function of culture age, environmental conditions, and experimental techniques and thus depends on the time

Table 7-7. ANOVA Table Comparing 2,4,6-TCP Flow-through and Static-Replacement Paired Tests Conducted During the Same Time Period. Data entered were $\log_{10}$ LC50's generated by quadratic regression with arc sin square root transformation.

Dependent Variable: $\log_{10}$ LC50

Source	df	Sum of Squares	Mean Square
Model	13	1.50256427	0.11558187
Error	41	0.32571568	0.00794428
Corrected Total	54	1.82827995	

Source	df	Type I SS	F Value	PR > F
Type	1	0.00002411	0.00	0.9563
Date	6	1.45874967	30.60	0.0001
Type·Date	6	0.04379048	0.92	0.4915

Tests of Hypotheses Using the Type IV MS for Type·Date as an Error Term

Source	df	Type IV SS	F Value	PR > F
Type	1	0.00176607	0.24	0.6403
Date	6	1.44327740	32.96	0.0002

Table 7-8. Nested ANOVA to Determine Between Tests and Within Test Variation for 2,4,6-TCP Tests. Data entered were $\log_{10}$ LC50's generated by quadratic regression with arc sin square root transformation.

Paired Flow-through and Static-Replacement Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	54	1.82828	0.03386	0.03535	100.00
Test No.	13	1.50256	0.11558	0.02741	77.53
Error	41	0.32572	0.00794	0.00794	22.47
Mean		0.308557			
Standard Deviation		0.089131			
Coefficient of Variation		0.288863			

Flow-through Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	26	0.76598	0.02946	0.03222	100.00
Test No.	6	0.61833	0.10306	0.02484	77.09
Error	20	0.14765	0.00738	0.00738	22.91
Mean		0.309231			
Standard Deviation		0.085920			
Coefficient of Variation		0.277851			

Static-Replacement Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	27	1.06228	0.03934	0.04320	100.00
Test No.	6	0.88421	0.14737	0.03472	80.37
Error	21	0.17807	0.00848	0.00848	19.63
Mean		0.307906			
Standard Deviation		0.092084			
Coefficient of Variation		0.299066			

period(s) in which tests are conducted. When tests are conducted during a single time period, under the same experimental conditions and using organisms from the same group of stock cultures, the results appear as a single large test with massive replication. If tests must be conducted during the same time period, then stock cultures of various ages should be used and tests should be conducted in separate growth chambers so that between tests variability approximates that for tests conducted at different times.

7.3. O-Cresol

Concentrations for paired flow-through and static-replacement tests were adjusted during the study period to obtain the best estimate of the LC50 and EC50's. Paired tests conducted during the same time period had the same concentrations. The concentration ranges, excluding the control, were 56-1000 mg/L, 45-800 mg/L, 81-700 mg/L, and 84-625 mg/L for tests 42-43, 51-52, 60-61, and 70-71, respectively.

The static-replacement and flow-through test mean LC50's were 540 and 464 mg/L, respectively; the ANOVA indicated these were statistically different at the 0.27 probability level (Table 7-9). However, the high volatility of o-cresol suggests that there should be a greater difference between static-replacement and flow-through tests. This lack of difference in LC50's between test types also occurred with 2,4,6-TCP (Section 7.2), but, as noted previously, the contract did not allow research to determine the reason. It is possible that the replacement schedule was sufficient to prevent a significant decline in the concentrations in the static-replacement test chambers.

The overall mean LC50 based on the combined static-replacement and flow-through tests was 501 mg/L with a 95% confidence interval of 427 and 589 mg/L. In the log scale these limits are within 3% of the mean. Given the magnitude of the mean, confidence limits within 10% of the mean would probably be satisfactory. Three tests of one replicate each would be adequate to obtain this level of precision (Table 7-6). However, conducting tests with only one replicate is not recommended. Replicates should be used to allow calculation and comparison of LC50's and calculation of variances within tests. Additionally, confidence intervals for each test cannot be calculated without replication.

Because more tests were conducted than were actually required, according to the predictions calculated

Table 7-9. ANOVA Table Comparing O-Cresol Flow-through and Static-Replacement Paired Tests Conducted During the Same Time Period. Data entered were $\log_{10}$ LC50's generated by quadratic regression with arc sin square root transformation.

Dependent Variable: $\log_{10}$ LC50

Source	df	Sum of Squares	Mean Square
Model	7	0.19387378	0.02769625
Error	23	0.02931974	0.00127477
Corrected Total	30	0.22319352	

Source	df	Type I SS	F Value	PR > F
Type	1	0.03870274	30.36	0.0001
Date	3	0.10178716	26.62	0.0001
Type·Date	3	0.05338389	13.96	0.0001

Tests of Hypotheses Using the Type IV MS for Type·Date as an Error Term

Source	df	Type IV SS	F Value	PR > F
Type	1	0.03161614	1.78	0.2747
Date	3	0.10620705	1.99	0.2932

(Equations 6-11 and 6-12) and data in Table 7-10, the actual test data were used to verify the prediction. A random numbers table was used to construct ten sets of three test groups with one replicate each from LC50's calculated for each replicate of each test. The three LC50's were averaged and a standard error was calculated. The average confidence limits for the ten groups were within 9.5% of the mean, thus confirming the number of tests and replicates predicted (Table 7-6).

7.4. Ethylene Glycol

Initial range-finding tests indicated definitive test concentrations should range from 0 to 56,000 mg/L. The concentrations used in all tests were 0, 1792, 3136, 5600, 10,080, 13,440, 17,920, 23,520, 31,360, and 56,000 mg/L.

No flow-through tests were conducted using ethylene glycol because of the relatively high effect thresholds. The amount of chemical used to produce the required concentration range in flow-through tests was cost-prohibitive. However, static-replacement tests were conducted to determine the LC50 and EC50's. The mean LC50 based on five 9-concentration, 4-replicate tests was 25,760 mg/L. The minimum and maximum LC50's generated were 17,006 and 40,139, respectively (Table 7-3). The 95% confidence limits for the mean LC50 were 16,360 and 40,560, which were within 4% of the mean; all of the LC50's generated with replicates pooled fell within these confidence limits.

The nested ANOVA indicated that 68% of the variation in ethylene glycol experiments occurred among tests as opposed to 32% which occurred within tests (Table 7-11). Calculations using Equations 6-11 and 6-12 indicated that to insure confidence limits within 25% of the mean, in log units, three tests with one replicate would be sufficient. However, the use of only one replicate per test is not recommended. A minimum of two replicates should be used to provide checks and to compare LC50's. The replicates can be pooled if no differences are noted in the replicate LC50's or raw data. Furthermore, with an additional replicate, Equation 6-12 indicates that the confidence limits should decrease to within 10% of the mean LC50 (Table 7-6).

7.5. Di(2-ethylhexyl)phthalate

Initial range-finding tests indicated the upper concentration for definitive tests should be approximately

Table 7-10. Nested ANOVA to Determine Between Tests and Within Test Variation for O-Cresol Tests. Data entered were $\log_{10}$ LC50's generated by quadratic regression with arc sin square root transformation.

Paired Flow-through and Static-Replacement Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	31	0.22490	0.00725	0.00790	100.00
Test No.	7	0.19507	0.02787	0.00666	84.26
Error	24	0.02983	0.00124	0.00124	15.74
Mean		2.699667			
Standard Deviation		0.035257			
Coefficient of Variation		0.013060			

Flow-through Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	15	0.09056	0.00604	0.00726	100.00
Test No.	3	0.07668	0.02556	0.00610	84.06
Error	12	0.01388	0.00116	0.00116	15.94
Mean		2.666768			
Standard Deviation		0.034010			
Coefficient of Variation		0.012753			

Static-Replacement Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	15	0.09971	0.00665	0.00798	100.00
Test No.	3	0.08376	0.02792	0.00665	83.33
Error	12	0.01595	0.00133	0.00133	16.67
Mean		2.732566			
Standard Deviation		0.036461			
Coefficient of Variation		0.013343			

Table 7-11. Nested ANOVA to Determine Between Tests and Within Test Variation for Ethylene Glycol Static-Replacement Tests. Data entered were $\log_{10}$ LC50's generated by quadratic regression with arc sin square root transformation.

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	18	0.56084	0.03116	0.03490	100.00
Test No.	4	0.40394	0.10099	0.02369	67.89
Error	14	0.15690	0.01121	0.01121	32.11
Mean		4.410965			
Standard Deviation		0.105864			
Coefficient of Variation		0.024000			

200,000 mg/L. Given this extremely high concentration range, and consequently the high mortality threshold concentration, mortality tests were not conducted. Therefore, the concentration ranges were set to provide data on reproduction and dry weight.

8.0 REPRODUCTION DATA ANALYSIS

An EC50 based on reproduction could not always be calculated using the concentration necessary for assessment of the LC50's. However, it was possible to calculate EC50's based on reproduction for some tests (Table 8-1). Two methods were used to calculate EC50's (Section 6.2). A comparison of the EC50's generated by the two methods indicated that the first method, based on frond numbers, yielded a lower EC50 in 92% of the cases than the second, based on the growth rate constant (Table 8-1). However, comparison of the coefficients of determination and the variance components of the two test types indicated less variation and thus more precision when the growth rate constant was used. Therefore, subsequent analyses were based on EC50's calculated using the growth rate constant.

8.1. $CuSO_4$

The concentrations used to assess the EC50's based on reproduction were the same as those used for calculation of the LC50's (Section 7.1). As with the LC50, the paired static-replacement tests yielded a higher EC50 ($\bar{x}$ = 3.51 mg/L) than the flow-through tests ($\bar{x}$ = 2.21 mg/L). Sequestering of a portion of the Cu^{++} by the fronds was offered as a possible explanation (Section 7.1). However, an ANOVA indicated that the static-replacement and flow-through test EC50's were not statistically different (Table 8-2), due to the relatively large variation within tests.

Variation within tests is due largely to the reproductive nature of the organisms. The new fronds form inside two small pockets on each side of the mother frond (Hillman 1961a). It is impossible to determine the stage of development of these fronds without harming the frond. Therefore, fronds selected for the tests may have daughter fronds ready to come forth or may have no daughter fronds. If daughter fronds are present, the generation time is shorter for the first generation, and more mother fronds are available to produce additional fronds. This effect is multiplied after several generations and results in different numbers of fronds in each test at the end of the test period, even if there is no effect of the

Table 8-1. Comparison of EC50's from Quadratic Regression for Paired Flow-through and Static-Replacement Tests: Square Root Transformation versus Growth Rate Constant (K) Transformation.

Toxicant	Test No. and Type[1]		EC50[2] $\sqrt{\text{fronds}}$	EC50[2] K
$CuSO_4$	13	F	0.80	2.32
	14	S	0.27[1]	2.23
	32	F	1.29	2.67
	33	S	0.81	2.98
	46	F	0.69	1.88
	47	S	2.08	4.10
	48	F	0.09	1.32
	49	S	2.60	6.03
	85	F	1.29	2.34
	86	S	1.58[3]	3.68
2,4,6-TCP	18	F	<0.01[3]	0.02[3]
	19	S	0.02	0.08
	35	F	<0.01[3]	0.02[3]
	36	S	<0.01[3]	0.01[3]
	54	F	<0.01[3]	0.02[3]
	55	S	0.23[4]	0.11
	64	F	<0.01[3]	0.03[3]
	65	S	1.78	0.95
	74	F	<0.01	0.03
	75	S	0.04[3]	0.14[3]
	88	F	0.02	0.10
	89	S	<0.01	0.03
	94	F	0.19[3]	0.17[3]
	95	S	--	--

Table 8-1 (Continued).

Toxicant	Test No. and Type[1]		EC50[2] $\sqrt{\text{fronds}}$	EC50[2] K
o-cresol	10	F	82.5	143.7
	11	S	230.5	260.4
	42	F	90.5	158.4
	43	S	135.5	193.1
	51	F	101.6	155.6
	52	S	168.2	237.8
	60	F	104.3	158.5
	61	S	286.2	370.1
	70	F	118.5	139.1
	71	S	137.4	230.9
ethylene glycol	37	S	13320	17140
	39	S	8950	15310
	45	S	10680	18970
	50	S	20000	27460
	66	S	35980	13160
di(2-ethylhexyl) phthalate	40	S	171.5[5]	735.1
	41	S	117.6	663.5
	44	S	502.6	7492.1
	53	S	569.5	2495.6
	63	S	3071.2	7469.9
	90	S	136.3	408.3
	93	S	365.1	5489.5

[1] Coupled entries indicate paired tests conducted during the same time period.
F = flow-through test
S = static-replacement test

[2] $\sqrt{\text{fronds}}$ = square root of total fronds Day 7

$$K = \frac{\log_{10}(\text{total fronds Day 7}) - \log_{10}(\text{total fronds Day 0})}{7}$$

[3] one replicate
[4] average of two replicates
[5] excluding conc = 6750 (otherwise EC50 = 0.84)

Table 8-2. ANOVA Table Comparing $CuSO_4$ Flow-through and Static-Replacement Paired Tests Conducted During the Same Time Period. Data entered were log_{10} EC50's generated by quadratic regression with growth rate constant transformation.

Dependent Variable: log_{10} EC50

Source	df	Sum of Squares	Mean Square
Model	9	0.94449280	0.10494364
Error	28	0.27369970	0.00977499
Corrected Total	37	1.21819250	0.02763584

Source	df	Type I SS	F Value	PR > F
Type	1	0.37989297	38.86	0.0001
Date	4	0.18651996	4.77	0.0046
Type·Date	4	0.37797986	9.67	0.0001

Tests of Hypotheses Using the Type IV MS for Type·Date as an Error Term

Source	df	Type IV SS	F Value	PR > F
Type	1	0.38353116	4.06	0.1142
Date	4	0.10944848	0.29	0.8714

toxicant. For example, the static-replacement controls over all paired tests varied from 36 to 159 fronds per chamber. The mean was 100 with a standard error of 8.3. The flow-through tests averaged 138 fronds with a standard error of 13.3 and ranged from 41 to 223 fronds per chamber.

The number of tests and replicates needed to obtain confidence limits within 10%, 25%, and 50% of the mean were calculated using Equations 6-11 and 6-12 and the data from the nested ANOVA's (Table 8-3). Although the ANOVA testing for differences between types (Table 8-2) indicated significant differences only above the 0.10 probability level, the predictions based on the separated static-replacement and flow-through tests probably should be used, as the predictions with the data pooled appear biased by the static-replacement data. Additionally, static-replacement and flow-through tests indicate opposite distributions of the variation (Table 8-3). In flow-through tests the within test variation is approximately 3X the between tests variation while in static-replacement tests the opposite appears true.

The predictions of the number of tests and replicates needed to yield confidence limits within 25% of the mean EC50 were the most reasonable (Table 8-4). To decrease the confidence limits to within 10% of the mean would not be cost-effective.

$CuSO_4$ was also used to determine if the number of fronds per test chamber had a significant effect on growth rates in static-replacement tests. Three sets of paired tests with four replicates each were conducted, and an ANOVA was calculated using EC50's for each replicate. The ANOVA indicated a significant difference between 4-frond and 12-frond chambers at the 0.60 probability level. The mean EC50 for both 4 and 12-frond tests was 2.75 mg/L. The 12-frond tests had slightly higher between tests variation (78%) in the replicate EC50's than the 4-frond tests (61%), based on the nested ANOVA variance components.

8.2. 2,4,6-TCP

The same concentration ranges that were used for the LC50 calculations were used to calculate the EC50's for 2,4,6-TCP (Section 7.2). Although the quadratic regression models fit the data relatively well, the solutions to the regression equations often yielded imaginary roots. The imaginary roots occurred when the entire curve was above or below the 50% effect level. The imaginary roots are, of course, completely invalid for this type of analysis and were treated as missing values in the data analysis.

Table 8-3. Nested ANOVA to Determine Between Tests and Within Test Variation for $CuSO_4$ Tests. Data entered were log_{10} EC50's generated by quadratic regression with growth rate constant transformation.

Paired Flow-through and Static-Replacement Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	37	1.21819	0.03292	0.03489	100.00
Test No.	9	0.94449	0.10494	0.02511	71.98
Error	28	0.27370	0.00977	0.00977	28.02
Mean		0.450277			
Standard Deviation		0.098869			
Coefficient of Variation		0.219573			

Flow-through Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	17	0.24780	0.01458	0.01528	100.00
Test No.	4	0.10490	0,02622	0.00428	28.04
Error	13	0.14290	0.01099	0.01099	71.96
Mean		0.344883			
Standard Deviation		0.104844			
Coefficient of Variation		0.304000			

Static-Replacement Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	19	0.59050	0.03108	0.03527	100.00
Test No.	4	0.45970	0.11493	0.02655	75.28
Error	15	0.13080	0.00872	0.00872	24.72
Mean		0.545132			
Standard Deviation		0.093381			
Coefficient of Variation		0.171299			

Table 8-4. Prediction of Minimum Number of Tests and Replicates Necessary to Obtain Confidence Limits Within 10%, 25%, and 50% of Mean at a 95% Probability Level (using $\log_{10}$ EC50's from quadratic regression with growth rate constant transformation for paired flow-through and static-replacement tests only).

Toxicant	Test Type	10%		25%		50%		T^2	s^2	$\bar{x}$
		k	r	k	r	k	r			
$CuSO_4$	F & S	~50	1	10	1	5	2	0.0251	0.0098	0.4503
	F	17	40	5	21	3	22	0.0043	0.0110	0.3449
	S	~38	10	9	2	4	4	0.0266	0.0087	0.5451
2,4,6-TCP	F & S	~55	102	11	74	5	16	0.1837	0.6236	-1.1780
	F	2	3711	2	594	2	149	0	1.1784	-1.6011
	S	>120	-	~45	11	13	13	0.4966	0.3461	-0.8757
o-cresol	F & S	4	3	3	1	3	1	0.0185	0.0043	2.2849
	F	2	7	2	2	2	1	0	0.0041	2.1794
	S	4	1	3	1	2	2	0.0144	0.0045	2.3905
ethylene glycol	S	3	1	2	7	2	1	0.0124	0.0095	4.2345
di(2-ethylhexyl) phthalate	S	11	35	4	6	3	1	0.2385	0.1652	3.3138

F = flow-through test
S = static-replacement test
k = number of tests
r = number of replicates

T^2 = estimation of τ^2 which is between test variation
s^2 = estimation of σ^2 which is between replicates within test variation
$\bar{x}$ = mean $\log_{10}$ (EC50)

Because of the number of replicates for which an EC50 could not be calculated, the data set for assessing means, confidence limits, and differences between types is limited. Therefore, all conclusions regarding reproduction in 2,4,6-TCP tests should be considered carefully. The following analysis of the data is presented as a best estimate of the effect of 2,4,6-TCP on reproduction.

The mean EC50's for static-replacement and flow-through TCP tests were 0.13 and 0.03 mg/L, respectively. The ANOVA testing for differences between static-replacement and flow-through tests indicated no significant difference until the 0.10 probability level (Table 8-5). Assuming acceptance of the 0.05 level as the breakpoint for determining if the two types of tests were different, the EC50's for the two test types can be pooled. Pooling is preferable because of the limited amount of valid replicate data for TCP. The pooled data ranged from EC50's of 0.01 to 0.95 mg/L. The 95% confidence limits about the pooled mean of 0.07 mg/L were 0.02 and 0.19 mg/L. The predicted number of tests and replicates were high even at the 25% and 50% levels (Table 8-4). The nested ANOVA showed approximately 3X more variation between replicates than between tests (Table 8-6), thus indicating the need for high replication. However, this may be an artifact of the data because of the few replicate EC50's within tests.

8.3. O-Cresol

The concentration ranges used to calculate EC50's were the same as those used in the LC50 calculations (Section 7.3). EC50's for paired static-replacement and flow-through tests were statistically different at the 0.05 probability level (Table 8-7). The mean EC50's were 245 and 151 mg/L for static-replacement and flow-through tests, respectively. In all paired tests the static-replacement EC50 was higher than the corresponding flow-through test EC50 (Table 8-1), due possibly to the volatility of o-cresol. However, the LC50's for o-cresol static-replacement and flow-through tests were statistically different at or above the 0.27 probability level (Table 7-9). The paired tests yielded LC50's that were almost identical in one case (tests 42-43), and in another the flow-through test LC50 was higher than the static-replacement test LC50 (tests 51-52, Table 7-3). Thus volatility may not be the cause of the statistically different EC50's for o-cresol.

Static-replacement test EC50's ranged from 193 to 370 mg/L o-cresol (Table 8-1). The 95% confidence limits were

Table 8-5. ANOVA Table Comparing 2,4,6-TCP Flow-through and Static-Replacement Paired Tests Conducted During the Same Time Period. Data entered were $\log_{10}$ EC50's generated by quadratic regression with growth rate constant transformation.

Dependent Variable: $\log_{10}$ EC50

Source	df	Sum of Squares	Mean Square
Model	11	10.77867574	0.97987961
Error	12	7.48257155	0.62354763
Corrected Total	23	18.26124729	

Source	df	Type I SS	F Value	PR > F
Type	1	3.06962659	4.92	0.0465
Date	5	5.50123692	1.76	0.1948
Type·Date	5	2.20781223	0.71	0.6286

Tests of Hypotheses Using the Type IV MS for Type·Date as an Error Term

Source	df	Type IV SS	F Value	PR > F
Type	1	1.75579474	3.98	0.1027
Date	5	2.11242792	0.96	0.5187

Table 8-6. Nested ANOVA to Determine Between Tests and Within Test Variation for 2,4,6-TCP Tests. Data entered were $\log_{10}$ EC50's generated by quadratic regression with growth rate constant transformation.

Paired Flow-through and Static-Replacement Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	23	18.26125	0.79397	0.80728	100.00
Test No.	11	10.77868	0.97988	0.18373	22.76
Error	12	7.48257	0.62355	0.62355	77.24
Mean		-1.177989			
Standard Deviation		0.789650			
Coefficient of Variation		-0.670338			

Flow-through Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	9	5.22965	0.58107	1.17841	100.00
Test No.	5	0.51602	0.10320	-0.68923	0.0
Error	4	4.71363	1.17841	1.17841	100.00
Mean		-1.601146			
Standard Deviation		1.085545			
Coefficient of Variation		-0.677980			

Static-Replacement Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	13	9.96197	0.76631	0.84270	100.00
Test No.	5	7.19303	1.43861	0.49659	58.93
Error	8	2.76894	0.34612	0.34612	41.07
Mean		-0.875734			
Standard Deviation		0.588318			
Coefficient of Variation		-0.671799			

Table 8-7. ANOVA Table Comparing O-Cresol Flow-through and Static-Replacement Paired Tests Conducted During the Same Time Period. Data entered were $\log_{10}$ EC50's generated by quadratic regression with growth rate constant transformation.

Dependent Variable: $\log_{10}$ EC50

Source	df	Sum of Squares	Mean Square
Model	7	0.52908719	0.07558388
Error	23	0.10087716	0.00438596
Corrected Total	30	0.62996435	

Source	df	Type I SS	F Value	PR > F
Type	1	0.33733763	76.91	0.0001
Date	3	0.10033407	7.63	0.0010
Type·Date	3	0.09141549	6.95	0.0017

Tests of Hypotheses Using the Type IV MS for Type·Date as an Error Term

Source	df	Type I SS	F Value	PR > F
Type	1	0.33094854	10.86	0.0459
Date	3	0.10001228	1.09	0.4714

156 to 387 mg/L. In the log scale, the confidence interval obtained using 4 tests with 4 replicates each was within 8% of the mean. The nested ANOVA (Table 8-8) and Equations 6-11 and 6-12 indicated 4 tests with 1 replicate would give confidence limits within 10% of the mean (Table 8-4).

The EC50's for flow-through tests ranged from 139 to 159 mg/L o-cresol (Table 8-1). The 95% confidence limits were 140 and 162 mg/L which were within 2% of the mean in the transformed units. These confidence intervals were obtained using 4 tests with 4 replicates each. The predictions indicated that 2 tests with 7 replicates each would be adequate to obtain confidence limits within 10% of the mean (Table 8-4).

8.4. Ethylene Glycol

Ethylene glycol was used only in static-replacement tests; no flow tests were conducted (Section 7.4). Concentrations used for calculation of the EC50's were the same as those used for the LC50's (Section 7.4). As with mortality, ethylene glycol did not appear to affect growth rates until it reached relatively high concentrations. In fact, the lowest concentration used (1800 mg/L) actually stimulated growth in most tests.

The EC50's averaged 17,200 mg/L and ranged from 13,160 to 27,460 mg/L (Table 8-1). An ANOVA indicated significant differences between test EC50's at the 0.004 probability level. The nested ANOVA indicated that the variance components were relatively evenly distributed with 57% between tests and 43% within tests (Table 8-9).

The 95% confidence limits about the mean were 12,000 and 24,000 mg/L. These limits are within 4% of the mean. The predicted number of tests and replicates needed to obtain confidence limits within 25% of the mean were 2 and 7, respectively. For confidence limits within 10% of the mean, 3 tests with 1 replicate were indicated (Table 8-4). As discussed previously, the use of only one replicate should be discouraged because it does not allow calculation of within test variation or a check on test results.

8.5. Di(2-ethylhexyl)phthalate

As with ethylene glycol, no flow-through tests were conducted using di(2-ethylhexyl)phthalate. The 7 static-replacement test EC50's ranged from 408 to 7492 mg/L with a mean of 2060 mg/L. The ANOVA indicated significant

Table 8-8. Nested ANOVA to Determine Between Tests and Within Test Variation for O-Cresol. Data entered were $\log_{10}$ EC50's generated by quadratic regression with growth rate constant transformation.

Paired Flow-through and Static-Replacement Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	31	0.65059	0.02099	0.02277	100.00
Test No.	7	0.54731	0.07819	0.01847	81.10
Error	24	0.10328	0.00430	0.00430	18.90
Mean		2.284915			
Standard Deviation		0.065601			
Coefficient of Variation		0.028711			

Flow-through Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	15	0.05318	0.00355	0.00407	100.00
Test No.	3	0.00431	0.00144	0.00659	0.0
Error	12	0.04887	0.00407	0.00407	100.00
Mean		2.179352			
Standard Deviation		0.063816			
Coefficient of Variation		0.029282			

Static-Replacement Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	15	0.24082	0.01605	0.01893	100.00
Test No.	3	0.18640	0.06213	0.01440	76.05
Error	12	0.05442	0.00453	0.00453	23.95
Mean		2.390479			
Standard Deviation		0.067339			
Coefficient of Variation		0.028170			

Table 8-9. Nested ANOVA to Determine Between Tests and Within Test Variation for Ethylene Glycol Static-Replacement Tests. Data entered were $\log_{10}$ EC50's generated by quadratic regression with growth rate constant transformation.

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	19	0.37785	0.01989	0.02184	100.00
Test No.	4	0.23572	0.05893	0.01236	56.61
Error	15	0.14213	0.00948	0.00948	43.39
Mean		4.234461			
Standard Deviation		0.097343			
Coefficient of Variation		0.022988			

Table 8-10. Nested ANOVA to Determine Between Tests and Within Test Variation for Di(2-ethylhexyl) phthalate Static-Replacement Tests. Data entered were $\log_{10}$ EC50's generated by quadratic regression with growth rate constant transformation.

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	24	9.03861	0.37661	0.40363	100.00
Test No.	6	6.06532	1.01089	0.23845	59.08
Error	18	2.97329	0.16518	0.16518	40.92
Mean		3.313817			
Standard Deviation		0.406427			
Coefficient of Variation		0.122646			

differences between tests ($P \leq 0.002$). The 95% confidence limits around the mean EC50 were 700 and 6000 mg/L, which are within 14% of the mean in log units. The variance components indicated 59% of the variation occurred between tests with 41% within tests (Table 8-10). The predictions, based on this variance breakout and Equations 6-11 and 6-12, indicate 4 tests with 6 replicates would be needed to keep confidence limits within 25% of the mean. To obtain confidence limits within 10% of the mean would not be cost-effective because 11 tests with 35 replicates each would be needed (Table 8-4).

9.0 DRY WEIGHT DATA ANALYSIS

Decrease in total weight per test chamber (replicate) was significant in almost all tests, even at the lowest concentration. The difference between control weights and the lowest concentration was so great that EC50's could not be calculated because determination of the LC50 was given priority in establishing concentration ranges; lower concentrations in flow-through tests would have made calculation of the LC50's impossible. If the concentration range were adjusted for dry weight, the range would not bracket the LC50. It was possible to extend the range of concentrations in some of the static-replacement tests. However, concentrations could be lowered only a limited amount and still be in the log series necessary for calculation of EC50's. An additional complication encountered in data analysis was that o-cresol and ethylene glycol were stimulatory at lower concentrations, causing increases in dry weight.

9.1. $CuSO_4$

Dry weight EC50's could not be calculated for $CuSO_4$ using either of the two methods discussed (Section 6.3). $CuSO_4$ was relatively toxic and quickly suppressed growth. The weight in the controls averaged 3X the weight in the lowest concentration tested (0.39 mg/L). An EC10 based on a 90% reduction in the control weight could be calculated for three of the static-replacement tests. The EC10's were 3.75, 2.78, and 2.17 mg/L for tests 49, 57, and 81, respectively, based on arc sin square root transformation. The lack of data made statistical comparison of static-replacement and flow-through tests impossible.

One set of static-replacement and flow-through tests was conducted with priority given to the concentrations for EC50 calculation based on dry weight. The concentrations used were 0, 0.112, 0.20, 0.36, 0.64, 1.12, and 2.0 mg/L for flow-through tests. Static-replacement tests had two additional concentrations (0.036 and 0.064 mg/L) to extend the range and hopefully to better bracket the EC50.

The test results were poor. Data points for the static-replacement test were scattered. The coefficient of determination (R^2) for the static-replacement test was 0.26 with a probability of a greater F at 0.013. The

calculated EC50 of 0.74 mg/L $CuSO_4 \cdot 5H_2O$ should be viewed with care due to the low R^2 value. The flow-through test results were also poor, although the coefficient of determination was higher (0.61) with the probability of a greater F at 0.0001. However, roots in the quadratic regressions for both tests were imaginary.

9.2. 2,4,6-TCP

An EC50 could be calculated for only four of the static-replacement tests using a $\log_{10}$ transformation of dry weight. The EC50's were 0.01, 1.63, 0.13, and 0.39 mg/L for tests 55, 65, 76, and 92, respectively. No EC50's could be calculated for flow-through tests.

EC10's based on arc sin square root transformation of dry weight in the test concentrations divided by the dry weight of controls could be calculated for five static-replacement tests and four flow-through tests. The static-replacement tests averaged 2.37 mg/L and ranged from 0.24 to 4.43 mg/L TCP. The flow-through tests averaged 2.82 mg/L with a low of 1.62 and a high of 5.64 mg/L TCP. Of all the above tests, two sets were paired; they yielded EC10's of 3.24 and 1.62 mg/L (tests 74-75) and 1.88 and 2.32 mg/L TCP (tests 94-95) for static-replacement and flow-through tests, respectively. Because of the limited data, no valid conclusions could be made about flow-through and static-replacement tests. In one case the static-replacement test EC50 was higher, while in the other the flow-through test EC50 was higher. The between tests variability was so high that resources would probably best be allocated by conducting several static-replacement tests rather than a few flow-through tests.

9.3. O-Cresol

Dry weight data for o-cresol tests were better suited for calculation of EC50's than data for the other toxicants. The concentration ranges for calculation of the EC50's were the same as those used for calculation of LC50's (Section 7.3). Quadratic regression with arc sin square root transformation gave a much higher R^2 value than did the log transformation (R^2 = 0.75 and 0.48, respectively), and therefore a better estimate of the EC50's (Table 9-1). Thus, as the ANOVA's based on this transformation were considered more appropriate, the following discussion is restricted to the arc sin square root transformed data.

Table 9-1. EC50's from Quadratic Regression Using $\log_{10}$ Dry Weight Transformation vs. Arc Sin Square Root of the Ratio Dry Weight to Control Weight Transformation for O-Cresol Paired Tests.

Test No. and Type	EC50 $\log_{10}$ transformation	EC50 arc sin $\sqrt{\ }$ transformation
42 F	113.9[4]	127.6[4]
43 S	97.7[4]	77.9[4]
51 F	152.1[4]	25.3[2]
52 S	121.7[4]	17.0[3]
60 F	96.8[4]	41.8[1]
61 S	134.4[4]	11.7[4]
70 F	97.8[4]	6.93[4]
71 S	174.6[4]	0.17[1]

[1]mean of 1 replicate

[2]mean of 2 replicates

[3]mean of 3 replicates

[4]mean of 4 replicates

The EC50's for static-replacement tests averaged 16.98 mg/L and ranged from 0.17 to 78 mg/L. This extremely wide range caused a large variance and consequently produced very wide confidence intervals. The 95% confidence intervals were 0.88 and 330 mg/L which were within 105% of the mean EC50.

Flow-through tests showed similar results. The mean EC50 was 32.4 mg/L; test EC50's ranged from 6.93 to 128 mg/L, with 95% confidence limits of 4.7 and 218 mg/L. These were within 55% of the mean in log units. The ANOVA indicated differences at the 0.11 probability level (Table 9-2). Thus, the pooled data (Table 9-3) should be used to predict the number of tests and replicates needed to yield confidence limits within 25% and 50% of the mean; 15 tests with 14 replicates each would be required for confidence limits within 25% of the mean, while 6 tests with 4 replicates would yield confidence limits within 50% of the mean. The pooled data based on 8 tests with an average of 3.25 replicates per test were within 40% of the mean EC50 (24.0 mg/L).

9.4. Ethylene Glycol

The same concentrations were used for calculations of EC50's and EC10's based on dry weight as were used for the LC50 calculations (Section 7.4). EC50's could be calculated for only two of the five static-replacement tests conducted; the EC50's for tests 39 and 45 were 9500 and 4400 mg/L, respectively. Ethylene glycol stimulated growth at the lower concentrations, producing dry weights above those in the control as well as scattering the data points. Thus, the correlation between concentration and dry weight was poor in most cases. Coefficients of determination ranged from 0.02 to 0.47 for those tests for which the EC50 could not be calculated.

9.5. Di(2-ethylhexyl)phthalate

EC50's and EC10's could not be generated for di-(2-ethylhexyl)phthalate. The toxicant was not soluble in or miscible with water; therefore, it formed oil droplets or globules, or completely covered the surface of the test chambers, depending on the concentration. At higher concentrations di(2-ethylhexyl)phthalate formed a thin layer completely across the beaker and coated the duckweed fronds. The oily coating did not evaporate under the drying conditions used (Section 5.4.2) and therefore increased dry weights. Consequently, it was not possible to make any correlations between dry weight and the effect of di-(2-ethylhexyl)phthalate on growth.

Table 9-2. ANOVA Table Comparing O-Cresol Flow-through and Static-Replacement Paired Tests Conducted During the Same Time Period. Data entered were $\log_{10}$ EC50's generated by quadratic regression with arc sin square root of the ratio dry weight to control weight transformation (control weight = mean dry weight at concentration 0).

Dependent Variable: $\log_{10}$ EC50

Source	df	Sum of Squares	Mean Square
Model	7	9.62867483	1.37552498
Error	18	3.22652895	0.17925161
Corrected Total	25	12.85520378	

Source	df	Type I SS	F Value	PR > F
Type	1	0.49060331	2.74	0.1154
Date	3	7.85501384	14.61	0.0001
Type·Date	3	1.28305768	2.39	0.1029

Tests of Hypotheses Using the Type IV MS for Type·Date as an Error Term

Source	df	Type IV SS	F Value	PR > F
Type	1	2.10953006	4.93	0.1129
Date	3	8.87448328	6.92	0.0733

Table 9-3. Nested ANOVA to Determine Between Tests and Within Test Variation for O-Cresol Tests. Data entered were $\log_{10}$ EC50's generated by quadratic regression with arc sin square root of the ratio dry weight to control weight transformation (control weight = mean dry weight at concentration 0).

Paired Flow-through and Static-Replacement Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	25	12.85520	0.51421	0.55334	100.00
Test No.	7	9.37552	1.37552	0.37409	67.61
Error	18	3.22653	0.17925	0.17925	32.39

Mean	1.378473
Standard Deviation	0.423381
Coefficient of Variation	0.307138

Flow-through Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	13	4.56571	0.35121	0.41011	100.00
Test No.	3	3.28583	1.09528	0.28213	68.79
Error	10	1.27988	0.12799	0.12799	31.21

Mean	1.505649
Standard Deviation	0.357755
Coefficient of Variation	0.237608

Static-Replacement Tests

Variance Source	df	Sum of Squares	Mean Squares	Variance Component	Percent
Total	11	7.79889	0.70899	0.84595	100.00
Test No.	3	5.85224	1.95075	0.60262	71.24
Error	8	1.94665	0.24333	0.24333	28.76

Mean	1.230101
Standard Deviation	0.493286
Coefficient of Variation	0.401012

10.0 ROOT LENGTH DATA ANALYSIS

The root length data were not useful in calculating EC50's because of the variability within and between tests. Normalization of the data was attempted using a $\log_{10}$ transformation of root length and concentration. All coefficient of determination (R^2) values for linear regression were less than 0.5, with 14 of 20 less than 0.1. The quadratic R^2 values were higher, with a mean R^2 of 0.77. Only six were less than 0.7. However, in most cases EC50's were difficult to determine because of the shapes of the curves.

One factor influencing the variability of the data was that when the frond died, the roots fell off or were broken off in handling. Detached roots were too fragile to be measured. Thus the root length data set was not complete. In addition, the number of roots measured in each chamber varied widely. For example, an o-cresol test (#43) had an average of 150 roots/chamber with a mean length of 21 mm at the lowest concentration, an average of 5.3 roots/chamber with a mean length of 6.7 mm at the median concentration, and an average of 6 roots/chamber with a mean length of 12 mm at the highest concentration. The control chambers averaged 113 roots/chamber with a mean root length of 21 mm. The curve for this test was a positive parabola. If taken literally the data would indicate that the toxicant had no effect at the lowest concentration, maximum effect near the median concentration, and a lesser effect as the concentration increased above the median.

The other data yielded similar results: 2,4,6-TCP, ethylene glycol, and di(2-ethylhexyl)phthalate tests produced both negative and positive parabolas and in one case an almost horizontal line. $CuSO_4$ was the only toxicant to produce a reasonable curve, with decreasing root length as concentration increased. The EC50 for these $CuSO_4$ tests based on 50% reduction of the mean control root length averaged 1.7 mg/L. This was similar to the EC50's for $CuSO_4$ based on reproduction.

Given the problems with measuring root length and analyzing the data, and the similarity in the EC50's for $CuSO_4$ based on reproduction and root length, reproduction rather than root length should be used as a means of assessing toxicant effects.

11.0 COST ANALYSIS

The relative costs for conducting a normal series of tests (7 concentrations x 4 replicates) were compared for flow-through and static-replacement tests. Costs were broken down into labor (estimated in man-hours) and non-labor (estimated in 1980 dollars). The cost analysis assumed that an experimenter conducting bioassays would already have typical laboratory equipment such as analytical balances, miscellaneous volumetric and standard glassware, magnetic stirrers, etc. The basic cost analysis was based on conducting a single test of 14-day duration as outlined in Section 5.0.

11.1. Culture Costs

Costs of maintaining stock cultures are the same regardless of the type of test conducted, because the same number of organisms is required for both flow-through and static-replacement tests. Initial cost of establishing the cultures and maintaining suitable conditions for their sustenance depends on the facilities available. Cultures should be maintained under conditions identical to test conditions. Therefore, it is assumed that space and temperature requirements for cultures are covered under costs for space and temperature control for the actual tests.

Normally three aquaria, approximately 16x25 cm (6 L), are sufficient to provide enough organisms for conducting a standard 7-concentration x 4-replicate test. Lighting for the aquaria costs approximately $100. Organisms for the tests can be obtained free. Cultures require approximately 21 days growth to provide enough organisms for the tests. Establishment and maintenance of the cultures, from the time they arrive until the time they are used in the tests, requires approximately 26 man-hours and $165 in non-labor items such as lights, aquaria, and chemicals.

11.2. Static-Replacement Test Costs

The requirements for static-replacement tests are nominal except for the costs of providing a temperature-controlled growth chamber (25°±3°). The necessary items

and approximate costs for conducting these tests are listed in Table 11-1. The cost of space for the tests depends on the facilities available. The actual space requirement is rather small (20 sq.ft.), assuming additional space is available for mixing chemicals, making assessments, etc. Cost of the space required was not determined because of the variables involved.

When the size of the static-replacement test is altered, the only real change in costs is in labor. Additional replicates or concentrations do not require significant increases in chemicals or other supplies. However, the assessment time changes considerably. For example, to conduct a 10-concentration x 10-replicate test, non-labor increased from $523 to $634 (21%) and labor increased from 53 man-hours to 100 man-hours (89%).

11.3. Flow-through Test Costs

Costs of conducting flow-through tests are considerably greater than for conducting static-replacement tests. The major cost increase is due to the amount of chemicals used in Hillman's M-medium, the labor necessary to mix these chemicals, increased costs of disposable items used with the dilutor panel, and the capital costs of equipment (Table 11-2). Additionally, approximately 192 sq.ft. are required. Part of this space could be used for static-replacement tests conducted concurrently with flow-through tests. The major capital equipment expense is the dilutor panel. Obviously this initial investment is lessened per test as more tests are conducted, but for one or few tests the cost may be prohibitive. The major increase in labor for a flow-through test is due to the time involved in mixing medium for test organisms and setup and breakdown of the dilutor panel.

11.4. Cost Comparisons

The cost differential between static-replacement and flow-through tests is due to additional equipment, nutrient solution, space, and labor required to clean, set up, and break down the flow-through tests. The costs of the test organisms and assessment are the same for both types of tests. Capital costs of flow-through tests are 11.5X the static-replacement costs when the entire cost of the dilutor system is considered. The cost differential decreases to 3.6X if the panel is not considered. Flow-through tests require approximately twice as much labor as static-replacement tests in order to set up, calibrate, and break

Table 11-1. Approximate Costs for Conducting a Single Static-Replacement Test.

Item	7 x 4[1] Quantity	7 x 4[1] Cost ($)	10 x 10[2] Quantity	10 x 10[2] Cost ($)
NON-LABOR				
Beakers (250 ml)	28	15	100	56
Chemicals[3]	-	243	-	243
Lights	2	50	4	100
Misc.	-	50	-	70
Culture[4]	-	165	-	165
Total		523		634
LABOR (Man-hours)				
Test setup	4.5		8.5	
Conduct/assess	9.5		28.25	
Test breakdown	4.75		11.75	
Measurements				
Root lengths	5.6		20.0	
Dry weight	2.5		6.0	
Culture[4]	26.0		26.0	
Total	52.85		100.5	

[1] 7 concentrations with 4 replicates each.
[2] 10 concentrations with 10 replicates each.
[3] Costs of minimum-size containers for reagents in Hillman's M-medium. Actual pro-rated cost of chemicals per test was $2.85.
[4] Establishment and maintenance.

Table 11-2. Approximate Costs for Conducting a Single Flow-through Test, 7 Concentrations with 4 Replicates Each.

Item	Quantity	Cost ($)
NON-LABOR		
Dilutor panel	1	5000
Aquaria	30	150
Lights	8	200
Troughs	2	300
Toxicant and diluent chambers	3 (min.)	600
Chemicals[1]	-	379
Misc. glassware	-	250
Tygon tubing	320 (ft.)	145
Cleaning chemicals	-	50
Deionized water	-	60
Culture[2]	-	165
Total		7299
LABOR (Man-hours)		
Test setup	21.0	
Conduct/assess	27.25	
Test breakdown	11.5	
Measurements		
Root lengths	5.6	
Dry weight	2.5	
Culture[2]	26.0	
Total	93.85	

[1] Costs of minimum-size containers for reagents in Hillman's M-medium. Actual pro-rated cost of chemicals per test was $155.

[2] Establishment and maintenance.

down the dilutor panel. Additional time is needed to mix the large volume of nutrient solution required by the flow-through tests.

The capital costs of the static-replacement test cannot easily be decreased; however, the labor costs could easily be reduced 21% by eliminating root length and dry weight measurements and by making assessments only on the final day of the tests. Detailed assessment on Days 1, 5, and 6 are not necessary, and if labor is a limiting factor, eliminating assessment on these days will save 3 man-hours per test.

Assuming a labor rate of $10/hr., the total cost of conducting a static-replacement test without making assessments on Days 1, 5, and 6, and not measuring dry weight or root length, would be approximately $930. A comparable flow-through test would cost approximately $8000. The costs of space and a controlled environment were not considered in either estimate.

The results of tests conducted on 2,4,6-TCP and o-cresol indicated no statistical difference between static-replacement and flow-through mortality tests. Although differences were detected for $CuSO_4$ tests at the 0.03 probability level, the additional costs of conducting flow-through tests would not be warranted and would be better allocated to conduct additional static-replacement tests.

12.0 COMPARISON OF MORTALITY, REPRODUCTION, DRY WEIGHT, AND ROOT LENGTH DATA

Mortality, reproduction, dry weight, and root length all were evaluated as parameters for determining the 50% effect concentrations for the toxicants used in this study (Section 5.2). Unfortunately, tailored tests were not conducted to evaluate the effect of the toxicants on each parameter. Mortality was designated the most important parameter; therefore, concentration ranges were established to obtain the most accurate projections of the LC50. The additional parameters were measured on all test organisms, but in some cases the data could not be used to calculate EC50's. In most cases the lowest concentration used in the mortality testing was at or above the upper range of concentrations needed to calculate EC50's based on dry weight, reproduction, and root length.

The root length data were the least useful. As previously discussed, the data could not be used to calculate an EC50 (Section 10.0). Data sets for each test were incomplete because duckweed roots fell off at the higher toxicant concentrations.

To determine the relative value of the other three parameters in calculating LC50's and EC50's, partial correlation coefficients were calculated (Section 6.5). Analyses conducted both by test and by toxicant yielded similar results. The data for tests pooled by toxicant are presented as an example of the results (Table 12-1). The partial correlation coefficients indicate that dry weight and reproduction (represented by the growth rate constant K) contained similar information about the effects of the toxicants on duckweed. The coefficients for $r_{y_1 y_4 . y_3}$ which measure the relationship of the log concentration and log dry weight without the effect of reproduction (K), indicate that log dry weight contains little information beyond that contained in K (Table 12-1). Similarly, the coefficients for $r_{y_1 y_4 . y_2}$ indicated little additional information in dry weight beyond that contained in the mortality data. These two sets of coefficients indicate that measuring dry weight in addition to mortality and reproduction (growth rates) does not contribute significant information about the effects of the toxicants on duckweed.

Considering the additional expense in labor and equipment necessary to measure dry weight (Section 11.0), it seems advisable to delete it from the data collection.

Table 12-1. Partial Correlation Coefficients to Determine the Correlation Between Concentration, Dry Weight, Reproduction, and Mortality.

Toxicant	Partial Correlation Coefficients[1,2]					
	$r_{y_1y_2 \cdot y_3}$	$r_{y_1y_3 \cdot y_2}$	$r_{y_1y_2 \cdot y_4}$	$r_{y_1y_4 \cdot y_2}$	$r_{y_1y_3 \cdot y_4}$	$r_{y_1y_4 \cdot y_3}$
$CuSO_4$	0.40	-0.46	0.80	-0.26	-0.80	-0.04[3]
2,4,6-TCP	0.58	-0.53	0.59	-0.36	-0.63	-0.53
o-cresol	0.33	-0.84	0.40	-0.72	-0.66	-0.24[4]
ethylene glycol	0.29	-0.64	0.70	-0.31	-0.80	0.09[5]
di(2-ethylhexyl) phthalate	0.38	-0.27	0.57	0.06[6]	-0.55	-0.25

[1] $y_1 = \log_{10}(\text{conc})$
$y_2 = \arcsin \sqrt{p}$
y_3 = growth rate constant K
$y_4 = \log_{10}(\text{dry weight})$

[2] significance probability = 0.0001 except where indicated otherwise

[3] significance probability = 0.41

[4] significance probability = 0.0011

[5] significance probability = 0.26

[6] significance probability = 0.29

Except for data analysis, the collection of reproduction data does not require any additional labor or non-labor costs beyond that for mortality data. Partial correlation coefficients indicate additional information beyond that in mortality is contained in the reproduction data (K).

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TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO.	2.	3. RECIPIENT'S ACCESSION NO.
EPA 560/6-81-003		

4. TITLE AND SUBTITLE
Comparison of Static-Replacement and Flow-through Bioassays Using Duckweed, Lemna gibba G-3

5. REPORT DATE
January 1981

6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)
John A. Davis, Ph.D.

8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Breedlove Associates, Inc.
618 Northwest 13th Avenue
Gainesville, Florida 32601

10. PROGRAM ELEMENT NO.
B2BL2S

11. CONTRACT/GRANT NO.
68-01-5776

12. SPONSORING AGENCY NAME AND ADDRESS
U.S. Environmental Protection Agency
Office of Pesticides and Toxic Substances
Washington, D.C. 20460

13. TYPE OF REPORT AND PERIOD COVERED
Final

14. SPONSORING AGENCY CODE

15. SUPPLEMENTARY NOTES

16. ABSTRACT

Static-replacement and flow-through tests were conducted using $CuSO_4 \cdot 5H_2O$, 2,4,6-trichlorophenol, and o-cresol to determine if they gave similar LC50's and EC50's for duckweed, Lemna gibba G-3. Static-replacement tests also were conducted using ethylene glycol and di(2-ethylhexyl) phthalate. Mortality, reproduction, dry weight, and root length were used to measure effect levels of the toxicants. LC50's and EC50's were calculated using quadratic regression with log transformation of the independent variable (concentration) and with several different transformations for the dependent variables. ANOVA's were used to test for differences between the two types of tests, tests within types, and replicates within tests. A procedure also was provided for estimating the number of tests and replicates necessary to obtain confidence limits within a given percentage of the mean.

Mortality and reproduction produced the best results. The highest variation occurred among tests, regardless of type, and the smallest variation was generally within tests (i.e. among replicates). Therefore, the best allocation of resources would be to replicate static-replacement tests in time, using four replicates per test.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
	bioassay, duckweed, Lemna gibba G-3, copper sulfate, 2,4,6-trichlorophenol, o-cresol, ethylene glycol, di(2-ethylhexyl)phthalate	

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
Release unlimited	unclassified	105
	20. SECURITY CLASS (This page)	22. PRICE
	unclassified	

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